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GABAERGIC MECHANISMS OF THE ANTIDEPRESSANT/ANTIPANIC DRUG PHENELZINE

by

KATHRYN GRACE TODD ©

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY
IN

PHARMACEUTICAL SCIENCES
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FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES
AND FACULTY OF MEDICINE (PSYCHIATRY)

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DEDICATION

This thesis is dedicated to the memory of Jean McKay Wylie Henning.

ABSTRACT

Studies were undertaken to investigate GABAergic mechanisms of phenelzine (PLZ), an antidepressant/antipanic drug reported to cause an elevation of brain GABA levels. The first group of experiments sought to clarify the role of benzodiazepine (BZD) binding sites in antidepressant action. Three classes of antidepressants were each administered to rats continuously for 21 days. Drug levels and BZD binding and 5-HT_2 binding parameters were assessed in cortex and hippocampus respectively. Results indicated that no drug class changed the density or affinity of BZD binding. All drugs tested, with the exception of fluoxetine (a novel antidepressant), decreased binding of radioligand to 5-HT_2 receptors. It has been suggested that a metabolite of PLZ, produced by the action of monoamine oxidase (MAO) on PLZ, may be responsible for its GABA-elevating effect, so the effects of prior administration of selective MAO-A and -B inhibitors on the GABA-elevating effects of PLZ were investigated. The results suggested that MAO-B may play a more important role in the GABA-elevating effects of PLZ than MAO-A. Two other hydrazine-containing MAO inhibitors, iproniazid and nialamide, were evaluated for their effects on brain GABA, alanine (ALA), catecholamine and 5-HT levels and on MAO, GABA-transaminase (GABA-T) and ALA-transaminase (ALA-T) activity. The data indicated that a free hydrazine function, such as that seen in PLZ, is important for elevation of GABA and ALA levels and for inhibition of GABA-T and ALA-T, but not for inhibition of MAO. PLZ was compared to a known GABA-T inhibitor, vigabatrin (VIG), with regard to inhibition of MAO, GABA-T and ALA-T and brain levels of GABA and ALA; studies were conducted with and without pretreatment with the nonselective MAO inhibitor tranylcypromine (TCP). Results from this series of investigations showed that while both PLZ and VIG had similar effects on GABAergic mechanisms, only PLZ inhibited ALA-T and elevated brain ALA levels. Pretreatment with TCP reversed the effects of PLZ, but not of VIG, on GABA and ALA, providing further evidence that a metabolite, produced by the action of MAO on PLZ, is responsible for the GABA-elevating action of PLZ.

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LIST OF ABBREVIATIONS

ALA	alanine
ALA-T	alanine-transaminase
AMI	amitriptyline
ANOVA	analysis of variance
B_{max}	maximum density of binding sites (in fmol mg^{-1} protein)
BZD	benzodiazepines
cAMP	cyclic adenosine monophosphate
CLG	clorgyline
CMI	clomipramine
CNS	central nervous system
CSF	cerebrospinal fluid
d	day
DEP	deprenyl
DA	dopamine
DMI	desmethylimipramine; desipramine
DOPAC	3,4-dihydroxyphenylacetic acid
ECD	electron capture detection(or)
eV	electron volt
FLU	flunitrazepam
FLUOX	fluoxetine
fmol	femtomole (10^{-15} moles)

g	gram
GH	growth hormone
GABA	γ-aminobutyric acid
GABA-T	GABA-transaminase
GC	gas chromatography
h	hour
5-HIAA	5-hydroxyindole-3-acetic acid
HP	Hewlett Packard
HPLC	high-pressure liquid chromatography
5-HT	5-hydroxytryptamine; serotonin
HVA	homovanillic acid
i.d.	internal diameter
IP	intraperitoneally
IPR	iproniazid
K_D	equilibrium dissociation constant (in nM)
kg	kilogram
l	litre
M	molar
NDCMI	N-desmethylclomipramine
MAO	monoamine oxidase
MAP	maprotiline
mg	milligram
min	minute

ml	millilitre
mm	millimetre
mM	millimolar
MS	mass spectrometry
NA	noradrenaline
ng	nanogram
nM	nanomolar
NIAL	nialamide
NFLU	norfluoxetine
PEA	2-phenylethylamine
PLZ	phenelzine
s	second
TCA	tricyclic antidepressant
TCP	tranylcypromine
TRIS	Tris buffer
VEH	vehicle
VIG	vigabatrin
μl	microlitre
μM	micromolar
°C	degrees Celsius

CHAPTER 1

General Introduction: GABA and the etiology and pharmacotherapy of depression and panic disorder.

1.1 INTRODUCTION

An early serendipitous finding on tuberculosis patients treated with the drug iproniazid showed these patients not only had an alleviation of their tuberculosis symptoms, but they also exhibited an elevation of mood (Selikoff *et al.,* 1952). It was subsequently reported that iproniazid was effective in inhibiting the catabolic enzyme monoamine oxidase (MAO), resulting in increased brain levels of the biogenic amines noradrenaline (NA) and serotonin [5-hydroxytryptamine, 5-HT] (Zeller *et al.,* 1952; Brodie *et al.,* 1956). Concurrently imipramine, a derivative of the neuroleptic chlorpromazine, was also found to have mood-elevating effects (Kuhn, 1957). It was reported that one of the effects of imipramine was the inhibition of the uptake of released NA back into the presynaptic nerve terminal, thereby increasing NA levels in the synaptic cleft (Glowinski and Axelrod, 1964; Iversen, 1971). Taken together, the results of these studies led to the formation of the biogenic amine theory of depression. That is, depression may be due to a functional deficiency of NA and/or 5-HT at certain synapses in the brain (Schildkraut, 1965; Lapin and Oxenkrug, 1969).

The biogenic amine theory of depression has received support from several investigations. It was reported that reserpine, a drug known to deplete monoamines, caused depressive symptoms when administered to humans (Klein, 1968). Further, metabolic precursors of NA and 5-HT were reported to attenuate, at least in some depressed patients, depressive symptoms, particularly when the precursors were co-administered with an inhibitor of MAO (Goldberg, 1980; van Praag, 1984; Young, 1984; Birkmayer *et al.,* 1984, Young, 1991). Another source of support comes from the reports that amphetamine, which not only releases catacholamines from the presynaptic terminal, but also blocks their uptake, causes a profound, albeit transient, elevation of mood (Satel and Nelson, 1989).

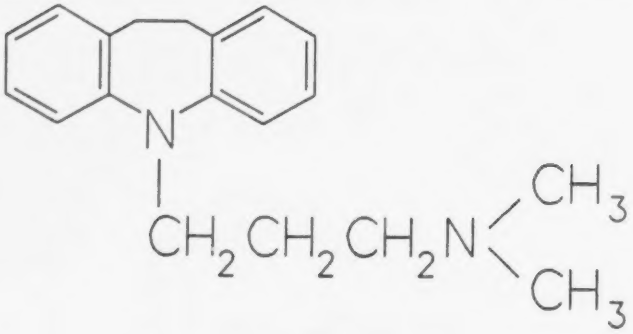
Although this theory of depression has indeed found support, there is also evidence which tends to argue against its validity. For example, although the biochemical changes associated with antidepressant treatment (inhibition of MAO and inhibiton of NA and 5-HT uptake) occur rapidly (within hours) after administration, clinical improvement requires several days to weeks (Oswald *et al.,* 1972; Quitkin *et al.,* 1986). Moreover, newer compounds developed, termed "novel" antidepressants, include drugs such as iprindole and mianserin that are neither MAO inhibitors nor NA or 5-HT uptake blockers (Freemand and Sulser, 1972; Sulser *et al.,* 1978). In an attempt to rectify these apparent contradictions, Ashcroft *et al.* (1972) proposed a modified amine hypothesis. They suggested that interactions among neurochemical systems as well as effects on the postsynaptic receptors were of importance in affective disorders. Consideration of the postsynaptic receptor was indeed relevant when it became apparent that treatment with many of the tricyclics, MAO inhibitors or novel antidepressants led to alterations of β-adrenergic or 5-HT_2 receptors (Charney *et al.*, 1981; Enna *et al.*, 1981; Baker *et al.*, 1989). These alterations may be evidenced as either changes in the function of second messenger systems subsequent to receptor activation (Sulser *et al.*, 1978) or as a decrease in the number (down-regulation) of receptors (Peroutka and Snyder, 1980).

Because of the inadequacies of the biogenic amine hypothesis, researchers have searched for other neurochemicals which may represent a locus for a mechanism of action common to the various types of antidepressants. One such neurochemical is γ-aminobutyric acid (GABA), and the remainder of this general introduction will provide a brief review of psychiatric drugs used to treat depression and of the possible involvement of GABA in the etiology and pharmacotherapy of depression and panic disorder.

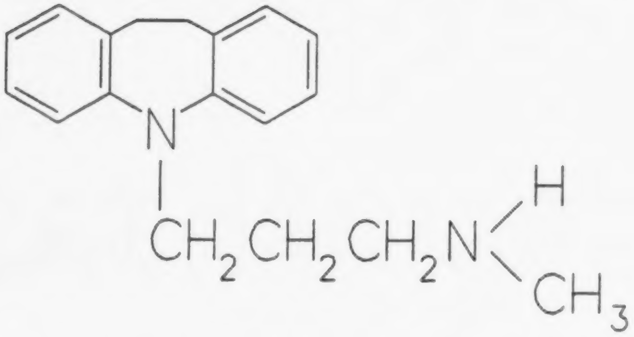
1.2. ANTIDEPRESSANT TREATMENTS

1.2.1. Tricyclic Antidepressants:

It was Kuhn's investigations in the late 1950's on the actions of several phenothiazine derivatives in depressed patients that lead the way in establishing the use of tricyclics as an effective pharmacological approach to the treatment of depression. Tricyclic antidepressants are so named because of their characteristic structure (Figure 1.), which has three fused rings. The prototypical tricyclic antidepressant is imipramine, with other newer tricyclics having variations in either the central ring or the side chain (Lader, 1980).



imipramine



desmethylimipramine

Figure 1. Examples of tricyclic antidepressants

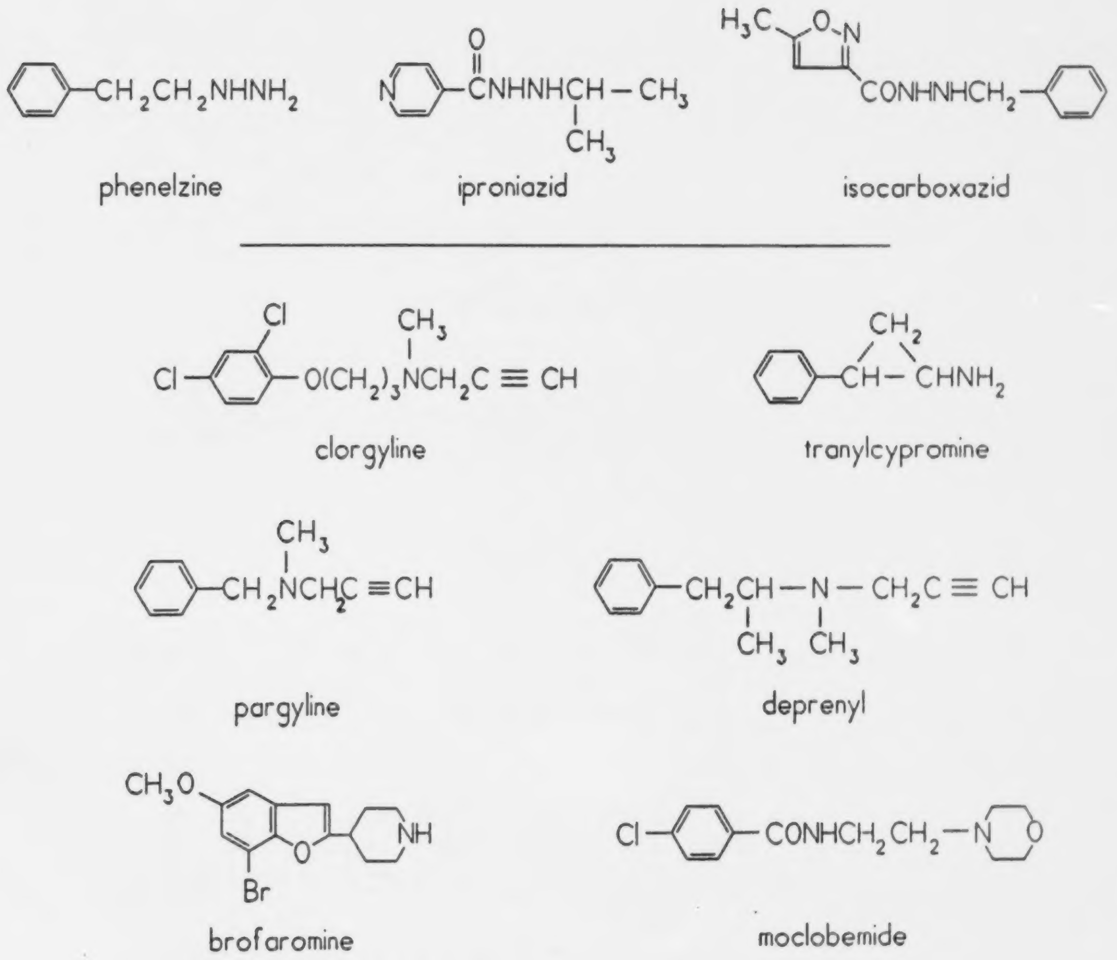
In humans, tricyclics are generally rapidly absorbed and extensively metabolized (Rudorfer and Potter, 1989). After oral administration, absorption is usually complete within 10 hours and maximal plasma concentrations obtained after 1 to 2 hours (Lader, 1980). Metabolism of tricyclics occurs *via* four main routes: 1. desmethylation of a side chain, 2. N-oxidation of a side chain, 3. hydroxylation at various positions of the ring structure, and 4. glucuronide formation (Rudorfer and Potter, 1989; Potter and Manji, 1990; Young, 1991). From these routes both inactive and active compounds are formed. For example, imipramine undergoes all of the above routes, and the N-desmethyl, N-oxide and 2-hydroxy metabolites are all pharmacologically active.

As with most pharmaceutical agents, there are side effects associated with the tricyclics. These effects are primarily due to antagonism at both peripheral nervous system and central nervous system (CNS) muscarinic and α-noradrenergic receptors. Anticholinergic effects include blurred vision, dry mouth, urinary retention, constipation, and excessive sweating (Abramowicz, 1980). Antagonism of α-noradrenergic receptors produces cardiovascular (e.g. hypo/hypertension, tachycardia, arrhythmias) and sedative effects as well as orthostatic hypotension. Photosensitivity and skin rashes have also been reported (Abramowicz, 1980; McDaniel, 1986; Arana and Hyman, 1991).

1.2.2. MAO Inhibitors:

As mentioned previously, it was the fortunate discovery that tuberculosis patients treated with MAO inhibitors had elevated mood that resulted in the subsequent use of this class of drug in the treatment of depression (Bosworth, 1959) (Figure 2.).

In humans, MAO is found in most tissue with the exception of the red blood cells and blood plasma (Blaschko, 1952). Most of the MAO is localized in the outer membrane of cellular mitochondria, and it is here that it carries out oxidative deamination reactions on a variety of monoamines (Fowler and Ross, 1984). MAO is now known to exist in two main forms, MAO-A and MAO-B, which are distinguished based on substrate preference and selectivity of inhibition (Johnson, 1968). More recent molecular biological (Weyler and Salach, 1985) and immunological studies (Denny *et al.*, 1982a) further support the hypothesis of two isozymes of MAO. The two isozymes have been shown to have different masses, with subunits of MAO-A having a mass of 63 kd while MAO-B subunits are of a mass 60 kd (Denny *et al.*, 1982a, 1982b). Peptide mapping studies combined with cDNA sequencing data have suggested that MAO-A and MAO-B are coded by different genes (Bach *et al.*, 1988; Hsu *et al.*, 1988; Powell *et al.*, 1988) that are situated proximal to one another in the Xp chromosomal region (Sims *et al.*, 1989). MAO-A preferentially metabolizes 5-HT and NA and is selectively inhibited by the MAO inhibitor clorgyline. MAO-B preferentially metabolizes β-phenylethylamine and is selectively inhibited by



phenelzine

iproniazid

isocarboxazid

clorgyline

tranylcypromine

pargyline

deprenyl

brofaromine

moclobemide

Figure 2. Examples of MAO inhibitors.

(-)-deprenyl (Johnson, 1968 Kinemuchi *et al.*, 1984).

In terms of distribution, most tissues, including the brain (Murphy and Donnelly, 1974) and the blood-brain barrier (Yu, 1984), contain a mixture of both isozymes. However, the relative distribution of MAO-A and -B in various brain regions has been shown to vary. Specifically, immunocytochemical studies using monoclonal antibodies have shown that MAO-A reactivity is concentrated in certain catecholaminergic areas such as the locus coeruleus, subcoeruleus and substantia nigra (Westlund *et al.*, 1985; Thorpe *et al.*, 1987). Autoradiographic procedures confirmed the abundance of MAO-A in the locus coeruleus and also found high concentrations in the paraventricular thalamus, raphé nuclei, solitary tract nucleus, inferior olives, interpeduncular nucleus, claustrum and peripheral tissues such as the liver, vas deferens, heart, superior cervical ganglia and the pancreas (Saura Marti *et al.*, 1990). Employing the same procedures, MAO-B was found to be differentially distributed in the ependyma, all circumventricular organs, raphé nuclei, paraventricular thalamus, posterior pituitary and liver (Saura Marti *et al.*, 1990). Placental tissue contains only the MAO-A isozyme (Salach and Detmer, 1979), while lymphocytes (Bond and Dundall, 1977) and platelets (Donnelly and Murphy, 1977) contain only MAO-B.

MAO inhibitors currently used clinically or experimentally are typically categorized in two ways: the mode of action (reversible or irreversible) and selectivity (selective for a given form of MAO or nonselective, inhibiting both

MAO-A and -B). The most commonly prescribed MAO inhibitors to date have been phenelzine (PLZ) and tranylcypromine (TCP) which are both irreversible and nonselective (Dubovsky, 1987). Recently, the clinical use of MAO inhibitors in the pharmacotherapy of depression and Parkinson's disease has been extended by the introduction of novel reversible and selective compounds. The irreversible MAO-A inhibitor clorgyline has been reported to be a good antidepressant (Murphy *et al.,* 1985), but can produce the "cheese effect" (see below). (-)-Deprenyl, an irreversible inhibitor of MAO-B, has not been a particularly successful antidepressant at doses where only MAO-B is inhibited (Mann *et al.,* 1989), but is now used extensively in the treatment of Parkinson's disease (Strolin Benedetti and Dostert, 1992). Reversible inhibitors of MAO-A include drugs such as moclobemide and brofaromine (Colzi *et al.,* 1993). Moclobemide is now clinically available as an antidepressant in several countries (Da Prada *et al.,* , 1990), while brofaramine is still undergoing clinical trial (Moller *et al.,* 1991).

The side effects of MAO-inhibiting drugs have been a problem, resulting in reduction in their clinical use. Of these side effects, orthostatic or postural hypotension is one of the most frequently described peripheral nervous system side effects and is most often a problem in patients with lower pretreatment blood pressure. Other effects include weight gain, sexual dysfunction, edema, insomnia, daytime sedation, monoclonus, reduction in rapid eye movement sleep and dry mouth (Wyatt *et al.,* 1971; Rabkin *et al.,* 1984; Murphy *et al.,* 1985).

The most serious, yet rare, side effect, is known as the "cheese effect". This side effect, with symptoms ranging from headache to a potentially lethal hypertensive episode, has occurred in patients concomitantly ingesting foods rich in sympathomimetic amines such as tyramine (aged cheese, red wine, chocolate, pickled or smoked meat or fish, fava beans, and yeast products). This effect is a consequence of the inactivation of intestinal MAO-A, which allows unmetabolised tyramine (due to the inhibition of MAO-A) to enter the circulation and exert an indirect pressor effect by releasing NA from storage vesicles (Sandler, 1981; Dostert, 1984). This release of NA may result in an exaggerated effect upon α-adrenergic receptors and dramatic elevation in blood pressure (Sandler, 1981). Health care professionals are able to circumvent this serious side effect by providing patients with a list of foods to avoid (McDaniel, 1986). Reversible MAO-A inhibitors by their very nature are much less likely to produce this side effect than are irreversible MAO-A inhibitors (Da Prada *et al.*, 1989; Nair *et al.*, 1993).

1.2.3. Atypical Antidepressants:

The significant side effect profiles associated with tricyclic and MAO-inhibiting antidepressants lead to a search for newer antidepressants with reduced side effects. These so-called atypical or novel antidepressants (Figure 3.) were of particular interest to researchers, as they are neither inhibitors of MAO nor, in several cases, potent blockers of uptake of NA, 5-HT or dopamine

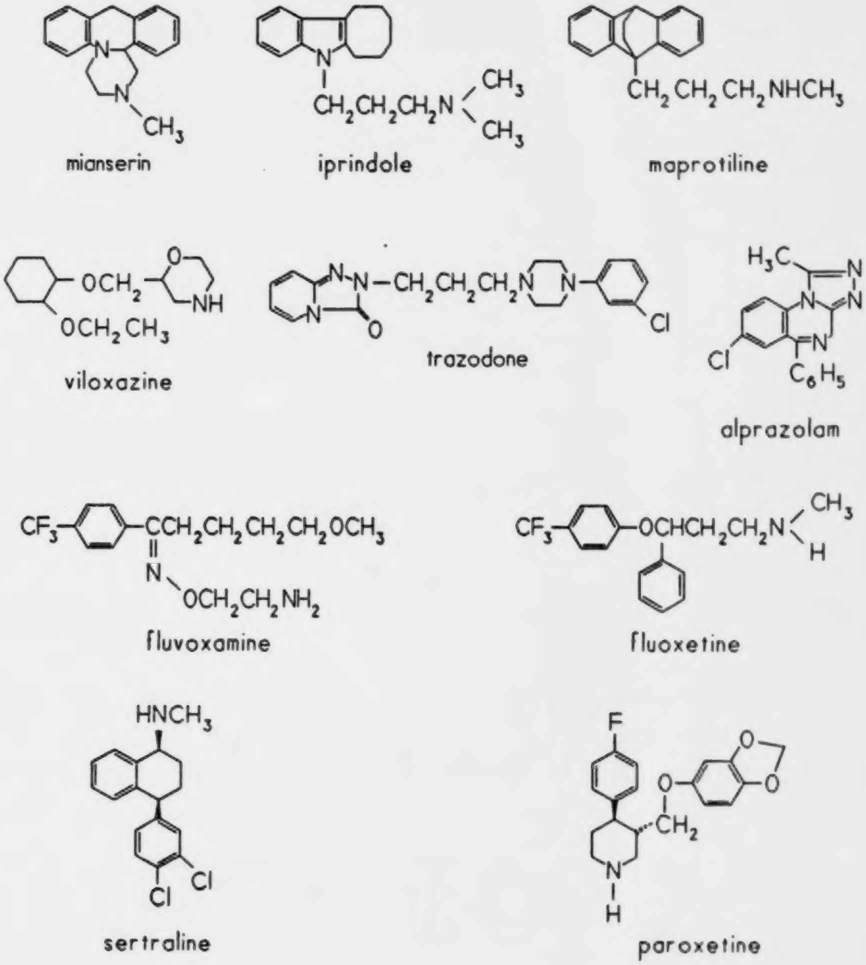

mianserin
iprindole
maprotiline
viloxazine
trazodone
alprazolam
fluvoxamine
fluoxetine
sertraline
paroxetine

Figure 3. Examples of novel antidepressants.

(Baldessarini, 1989).

Iprindole was the first atypical antidepressant to be tested clinically. This compound has a modified tricyclic structure with an indole group replacing two groups of the standard tricyclic structure, which apparently accounts for its lack of uptake blocking activity (Rudorfer and Potter, 1989). In clinical trials, iprindole was reported to have fewer side effects than tricyclic or MAO-inhibiting antidepressants; however, its efficacy as an antidepressant is still a matter of debate (Baldessarini, 1989).

Mianserin, a tetracyclic atypical antidepressant, is reported to have good antidepressant efficacy with few side effects (Damlouji *et al.*, 1985). Its acute neurochemical activity apparently involves the presynaptic blockade of α-adrenergic receptors, resulting in increased NA release (Rudorfer and Potter, 1989).

Another atypical antidepressant, trazodone, acts as a 5-HT agonist *in vivo*, and it is thought that m-chlorophenylpiperazine (m-CPP), a major metabolite of this drug, may in fact be the active agent (Potter and Manji, 1990).

Fluoxetine, a selective 5-HT uptake inhibitor, has become one of the most frequently prescribed antidepressants. Unlike the tertiary amine tricyclics (e.g. imipramine, amitriptyline, clomipramine), whose N-desmethylated metabolites are weaker inhibitors of 5-HT and stronger inhibitors of NA uptake than the parent drugs, fluoxetine's N-desmethylated metabolite is also a potent and selective 5-HT uptake inhibitor with an even longer half-life than that of the parent compound (Benfield *et al.,* 1986). Fluoxetine has been reported to lack anticholinergic, hypotensive and sedative side effects and as such is often the drug of choice to treat depressive symptoms (Schatzberg *et al.,* 1987).

The success of fluoxetine has led to the recent introduction of several other selective serotonin uptake inhibitors (SSUIs) as clinical antidepressants; these drugs include fluvoxamine, paroxetine, and sertraline (Feighner and Boyer, 1991; Warrington, 1992; Gram *et al.,* 1993). Similar to fluoxetine, and unlike the 5-HT uptake inhibiting tertiary amine tricyclics, the metabolites of these compounds retain the specific 5-HT uptake blockade of the parent compound or, at most, have very weak activity on NA uptake (Wong *et al.,* 1974; Claasen *et al.,* 1978; Squires, 1974; Hyttel, 1982; Baumann, 1992; Pinder and Wieringa, 1993).

1.3 GABA AND DEPRESSION/PANIC DISORDER AND ANTIDEPRESSANT/ANTIPANIC DRUGS

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the CNS: it is the primary transmitter in approximately 30% of central neurons (Sieghart, 1989). It exhibits a ubiquitous distribution in the brain, suggesting an important role in a wide variety of physiological mechanisms including the secretion of hormones (Racagni and Donoso, 1986) and control of cardiovascular functions, pain, anxiety, motor coordination, feeding and aggressive behavior (Krogsgaard-Larsen, 1988). Recently, there has also been the proposition that GABA may also play a role in psychiatric dysfunctions such as depression (Lloyd *et al.,* 1989). This possible involvement is discussed in detail in section 1.3.3.

GABA was first synthesized in 1883 and was known as a product of microbial and plant metabolism (Cooper *et al.,* 1991). Its possible role in the vertebrate nervous system had a less than auspicious beginning. Early investigators showed that GABA had an inhibitory action on the crayfish stretch receptor system (Bazemne *et al.,* 1957). However, its precise role remained unclear, and the compound was even denounced, as other evidence suggested "against it playing a role as an inhibitory transmitter in vertebrates" (Roberts, 1986). Fortunately, research continued on the vertebrate nervous system and GABA became accepted as a major inhibitory transmitter in the CNS. Concomitant with its acceptance, the biochemistry of GABA was elucidated.

1.3.1. Metabolism of GABA:

GABA is formed from L-glutamic acid *via* decarboxylation in a reaction catalyzed by L-glutamic acid decarboxylase (GAD) (reviews: Bradford, 1986; Cooper *et al.,* 1991). GAD catalyzes the removal of the γ-carboxyl group as CO_2 to produce the γ-amino acid. Immunohistochemical analysis showed GAD to be specifically localized in the nerve terminals of GABA-releasing neurons and thus a reliable chemical marker for GABAergic cells (McLaughlin *et al.*, 1975).

The rate of GABA synthesis in brain is a small proportion of total GAD activity, indicating that GAD operates at only a fraction of its maximal catalytic capacity (Martin and Rimval, 1993). Thus, the brain contains far more GAD than is required to meet the normal demand for new GABA. GAD requires pyridoxal phosphate as a cofactor for its action. At least 50% of GAD is present in brain as an apoenzyme (GAD without its bound cofactor), thereby providing a reservoir of inactive GAD that can be drawn on when additional GABA is required (Miller *et al.*, 1980). Recent active-site labelling experiments have shown that GAD is unusual, if not unique, among pyridoxal phosphate-dependent enzymes in that it is present in a large amounts in the form of apoenzyme.

Continuing along the metabolic chain to catabolism, GABA subsequently undergoes transamination by GABA-α-oxoglutarate transaminase (GABA-transaminase, GABA-T). This enzyme is found primarily in the grey matter of the CNS in the cellular mitochondria (Roberts, 1986). Transamination occurs within presynaptic GABA terminals as well as in surrounding glial cells, indicating that extracellular enzymatic breakdown is not the major mechanism of GABA inactivation (Krogsgaard-Larsen, 1988). GABA-T also employs pyridoxal phosphate as cofactor. However, unlike the binding to GAD, the cofactor binds in greater proportion to GABA-T, with a brain ratio of GABA-T/GAD activity typically greater than 1 (Cooper *et al.,* 1991). Moreover, the transamination of GABA by GABA-T is a reversible reaction. The products of the forward reaction are succinic semialdehyde and glutamic acid. Succinic semialdehyde dehydrogenase then catalyzes the oxidation of succinic semialdehyde to succinic acid which in turn is oxidized by the reactions of the Krebs cycle.

The termination of GABA transmission appears to be largely the result of an uptake mechanism in the form of a sodium-dependent pump. High and low affinity GABA uptake systems in brain tissue have been shown in both neuronal and glial cell preparations (Martin, 1976). The properties of the high affinity system may be summarized as follows (Erecinska, 1987):

1) exhibits Michaelis-Menton kinetics.
2) leads to accumulation of GABA within the cell at concentrations 3-4 orders of magnitude higher than outside.
3) is energy dependent.
4) requires sodium ions.

1.3.2. GABA Receptors:

Initially, GABA receptors were defined by their sensitivity to inhibition by two compounds: bicuculline and picrotoxin (Curtis *et al.,* , 1971). Bowery *et al.* (1981) further elucidated two GABA receptor subtypes based on their sensitivity to baclofen, a GABA agonist. Specifically, bicuculline-sensitive, baclofen-insensitive GABA receptors were defined as $GABA_A$ sites and bicuculline-insensitive, baclofen-sensitive receptors termed $GABA_B$ sites. Due to the wide variety of compounds available for labelling the $GABA_A$ site, the majority of investigations have concentrated on this receptor subtype.

Agonist activation of the $GABA_A$ receptor results in the opening of an integral chloride anion channel. *In vivo*, the change in chloride ion permeability typically results in hyperpolarization of the postsynaptic cell (postsynaptic inhibition) or depolarization of the presynaptic cell (presynaptic inhibition) (Cooper *et al.,* 1991).

Evidence has suggested that $GABA_A$ receptors may be further subdivided based on their sensitivity to benzodiazepines (BZDs) (Enna and Karbon, 1986). It has been postulated that although most BZD binding sites are associated with $GABA_A$ receptors (Farrant *et al.,* 1990), not all $GABA_A$ receptors have a BZD component (Unnerstall *et al.,* 1981). To more clearly elucidate the receptor subtypes, a number of GABA agonist ligands have been used, including muscimol, isoguvacine and 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridin-3-ol (THIP) (Beaumont *et al.,* 1977; Falch and Krogsgaar-Larsen, 1982). These studies have identified high, low and ultralow affinity $GABA_A$ binding sites. Other investigators identified two other possible components of the receptor complex: the chloride ion channel (identified using dihydropicrotoxin as the ligand, Ticku *et al.*, 1978) and a BZD binding site (identified using diazepam and flunitrazepam as ligands, Möhler and Okada, 1977; Enna and

Möhler, 1987; Martin, 1987; Pritchett, 1989). Other binding sites reported to coexist on the $GABA_A$ receptor complex are a barbiturate binding site, a pictrotoxin/t-butylbicyclophosphorthionate (TBPS) binding site, a steroid binding site and an ethanol binding site (Gee, 1988).

In general, the pharmacology of the $GABA_A$ receptor complex revealed by the aforementioned studies may be summarized as follows (Mohler and Okada, 1977; Ticku *et al.*, 1977; Krogsgaard-Larson, 1982; Martin, 1987; Gee, 1988):

GABA binding sites:

a) Agonists (e.g. GABA, muscimol) bind to the GABA recognition site and activate the chloride channel.

b) Antagonists (e.g. bicuculline) compete with GABA for its binding site and prevent chloride channel opening.

Benzodiazepine binding sites:

a) Agonists (e.g. diazepam, flunitrazepam) potentiate GABA's effects and increase the frequency of channel opening.

b) Inverse agonists (e.g. beta-carbolines) compete with BZD agonists but cause opposite effects; i.e. decrease the frequency of channel opening.

c) Antagonists (e.g. Ro15-1788) compete for agonist or inverse agonist binding sites and prevent their effects, but do not themselves have overt effects.

Chloride channel:

a) Picrotoxin and TBPS reduce chloride channel conductance by sterically hindering the entry of chloride across the ion channel. Binding sites for these compounds are thought to be located in close proximity to the chloride channel.

b) Barbiturates (e.g. pentobarbital) enhance GABA actions by increasing mean channel open lifetime. At higher concentrations barbiturates will enhance chloride ion conductance in the absence of GABA.

c) Steroids such as alphaxalone and 5-α-reduced metabolites of progesterone enhance chloride channel conductance.

$GABA_A$ receptors have been found postsynaptically (axo-somatic or axo-dendritic) and presynaptically (axo-axonic). Activation of the postsynaptic receptor leads to hyperpolarization, resulting in a decreased sensitivity of the neuron to excitatory input. Activation of the presynaptic receptor results in a net efflux of chloride ions, causing a partial depolarization and reduced release of neurotransmitter (presynaptic inhibition).

Recently, molecular cloning studies of the $GABA_A$ receptor have shown that there are at least three subunits that make up this receptor complex, including the α, β and γ subunits (Doble and Martin, 1992). Further, molecular biological techniques have revealed the presence of multiple isoforms of the subunits, each encoded by a separate gene (Luddens and Wisden, 1990). Different combinations of these isoforms making up the $GABA_A$ complex will lead to different functional receptors with unique pharmacological properties. Interestingly, investigations have shown that chronic treatment with $GABA_A$/BZD receptor ligands leads not only to functional alterations, but also to alterations in the expression of individual subunit isoform mRNAs (Gallager *et al.,* 1985; Kang and Miller, 1990). This avenue of research, as it progresses, will likely shed a great deal of light on the mechanisms of action of drugs proposed to act on GABAergic systems including antidepressant and antipanic drugs.

Although much less studied, the $GABA_B$ receptor subtype has also been labelled and characterized to some extent. Labelling of this receptor is most often accomplished with ^{3}H-baclofen. The $GABA_B$ receptor also appears to have multiple binding sites, with at least high and low GABA affinity sites which are calcium-dependent (Bowery *et al.,* 1984; Karbon *et al.,* 1983). In contrast to the $GABA_A$ receptor complex, $GABA_B$ receptors are not coupled with a BZD recognition site.

In summary, it is possible to differentiate $GABA_B$ from $GABA_A$ receptors based on their sensitivity to baclofen, insensitivity to bicuculline and the lack of association with the BZD receptor site.

1.3.3. GABA and Depression:

Whether there is a possible role of GABA in depression remains somewhat controversial. A number of studies have reported low GABA levels in the CSF (Gold *et al.,* 1980; Gerner and Hare, 1981; Kasa *et al.,* 1982) and blood plasma of patients diagnosed with depression (Berettini *et al.,* 1982; Petty *et al.,* 1990). These clinical findings were thought to reflect changes in brain GABA levels, and the search was on to isolate GABAergic mechanisms of depression and antidepressant treatments.

Animal studies on GABAergic mechanisms have primarily employed four models for depression: the removal of the olfactory bulb, producing deficits in passive avoidance in the rat (Lloyd *et al.*, 1989); the forced swimming test, inducing immobility, thereby putatively measuring learned helplessness in the rat (Petty, 1986); the reduction in paradoxical sleep in the rat (Lloyd *et al.*, 1983); and the antagonism of serotonin receptor-induced head twitches in the mouse (Singh *et al.*, 1986). In each of these behavioral models, the GABAmimetic progabide (active at both $GABA_A$ and $GABA_B$ receptors) reversed the behavioral deficits induced by the paradigms. In the olfactory bulbectomy paradigm, muscimol also significantly reversed the passive avoidance deficit (Lloyd *et al.*, 1983). Moreover, the GABAmimetics showed their effects acutely (after a single dose), whereas most conventional antidepressants required prolonged administration.

In the forced swim test (considered to be a model of learned helplessness), data indicated that the tricyclic antidepressant imipramine also reversed the behavioral deficit (Petty, 1986; Suryani-Cadotte *et al.*, 1990). Bicuculline injected intracerebrally was found to reverse imipramine action, and, if administered alone, induced a state of immobility (Petty, 1986). In this model of depression, bicuculline significantly reversed the action of both fengabine (mixed $GABA_A$- and $GABA_B$-mimetic) and imipramine (Lloyd *et al.*, 1987).

The behavioral data suggest that enhancing GABA activity has beneficial effects such as alleviating the behavioral deficits in what are considered possible animal models of depression. The biochemical investigations designed to delve further into mechanisms involving $GABA_B$ receptors are seemingly divided into two camps: studies that postulate changes in $GABA_B$ receptor number and support a GABAergic hypothesis for depression, and those who find no $GABA_B$ changes and therefore tend to refute the hypothesis.

Lloyd *et al.* (1985) have suggested that different "monoamine specific" and other classes of antidepressants have a common action that is more closely related to their clinical antidepressant effect than their actions at monoamine synapses. The common mechanism of action was postulated to be an upregulation of $GABA_B$ receptors. These investigators chronically administered a number of antidepressant compounds, including an NA uptake inhibitor (desipramine), a 5-HT uptake inhibitor (fluoxetine), a dopamine uptake inhibitor (nomifensine), a mixed uptake inhibitor (amytriptyline), an MAO inhibitor (pargyline), a GABAmimetic (progabide) and electroshock. All drug treatments and electroshock resulted in an increased number of $GABA_B$ receptors in the rat frontal cortex, but not in the hippocampus. $GABA_A$ receptor number was not changed in response to drug or electroshock administration. The mechanism for this up-regulation remains somewhat of an enigma, as brain GABA levels and GABA synthesis were found to be unchanged *ex vivo* or *in vitro* following clinically relevant doses of antidepressants (Lloyd and Pilc, 1984). Gray and Green (1987), in a similar experiment, also found $GABA_B$ receptor up-regulation following chronic antidepressant and electroshock treatment in mice.

In an investigation of chronic imipramine and baclofen treatment, Suzdak and Gianutsos (1986) concurred with the findings of Lloyd *et al.* (1984). Results of the former study showed no change in $GABA_B$ or β-adrenergic receptor binding following acute treatment of either drug. After chronic treatment (14 days) of baclofen, a down-regulation of β-adrenoreceptors and $GABA_B$ binding was reported which the authors suggested was an effect of the associations of the two receptor types. In contrast with baclofen, chronic imipramine treatment resulted in decreased β-adrenoreceptor binding but increased $GABA_B$ receptor binding. These data lead to the suggestion that activation of the $GABA_B$ receptor modulated existing noradrenergic input by "fine-tuning" post-synaptic noradrenergic stimulation and thus produced antidepressant effects. However, Lloyd *et al.* (1985) demonstrated that other antidepressants up-regulated $GABA_B$ binding in the absence of intact noradrenergic neurons. These findings suggested that there were independent noradrenergic and $GABA_B$ receptor effects of antidepressants. These data have yet to be reconciled and a mechanism for $GABA_B$ receptor up-regulation clearly elucidated.

Further studies have continued to implicate $GABA_B$ receptors in depression and antidepressant action (Gray *et al.*, 1987; Martin *et al.*, 1989).

Martin *et al* (1989) found down-regulation of $GABA_B$ receptors in the frontal cortex of olfactory bulbectomized rats and in models thought to induce learned helplessness (forced swim test). Moreover, GABA release was found to have decreased in the hippocampus. The down-regulation of $GABA_B$ receptors in the frontal cortex was reversed following antidepressant treatment with imipramine and desipramine, strongly suggesting a GABAergic mechanism of action.

Conversely however, there is also an abundance of data which refutes a GABAergic mechanism of action. In studies that employed similar methodologies to the aforementioned, $GABA_B$ receptors were found to be unchanged following chronic antidepressant treatments (Cross and Horton, 1987, 1988; McManus and Greenshaw, 1991; Monteleone *et al.,* 1990; Stocks *et al.,* 1990; Szekely *et al.,* 1987).

Cross and Horton (1987, 1988) found unaltered $GABA_B$ receptors in rat cortex after chronic (21 day) administration of the antidepressants desipramine and zimelidine both orally and intraperitoneally (IP). These authors did report, however, reduced 5-HT_2 binding sites in frontal cortex after both drugs were administered orally and with desipramine only after IP administration. The authors concluded that the effects of antidepressants on $GABA_B$ binding sites were less consistent than their effects on 5-HT_2 binding sites.

In an attempt to reconcile their conflicting results, Cross and Horton (1988) compared their methodologies with those of Lloyd *et al.* (1985), who had found up-regulation of $GABA_B$ binding following antidepressant treatment. Two methodological differences were obvious-- route of administration (osmotic minipumps versus IP injection) and time of decapitation after drug discontinuation.

Similarly, McManus and Greenshaw (1991) reported no changes in $GABA_B$ receptors following chronic (28 day) antidepressant treatment. These investigators employed osmotic minipumps for continual infusion of drug similar to Lloyd *et al.* (1985), further supporting Cross and Horton's findings. McManus and Greenshaw reported functional changes (down-regulation) in β-adrenoreptors following antidepressant treatments, which coincide with the findings of Suzdak and Gianutsos (1986), yet their results conflicted with the reports of the latter authors of changes in $GABA_B$ receptor number. McManus and Greenshaw concluded that their results did not support the hypothesis that chronic administration of antidepressants leads to an increase in the number of $GABA_B$ receptors.

In agreement with these findings of no change of $GABA_B$ receptor number are recent clinical studies. Monteleone *et al.* (1990) investigated the $GABA_B$ binding sites in healthy men and depressed patients by measuring plasma growth hormone (GH) responses to baclofen. This endocrine model is based on the finding that $GABA_B$ receptors in the hypothalamus were involved in the modulation of GH secretion. Therefore, measuring plasma GH levels in response to acute administration of baclofen ($GABA_B$ stimulation) was considered to reflect $GABA_B$ receptor functions. Results showed that there were no differences between control and depressed patients in their GH response to $GABA_B$ activation. The authors therefore concluded that their results did not support the proposal that GABAergic mechanisms are involved in depression and in the action of antidepressant drugs. They did suggest, however, that depressed patients could have impairments in central GABAergic transmission outside the hypothalamus. It is of interest that Paykel *et al.* (1991) found that fengabine, a GABA agonist which had been reported by Scatton *et al.* (1987) to upregulate $GABA_B$ receptors in rat brain, was ineffective as an antidepressant in the clinical setting.

Further data which serve to add fuel to the fire of this contentious issue are those relating to $GABA_A$ receptors. Although the number of studies is not as great as that focusing on $GABA_B$ receptors, this area is now receiving considerable attention. Stocks *et al.* (1990) studied amygdala and hippocampal tissue from depressed suicide victims. Their data indicated that the number and affinity of BZD receptor sites did not differ significantly between drug-treated and drug-free suicide victims and controls. Cross and Horton (1987) concurred with these results in their study that showed chronic (21 day) antidepressant treatment did not change the number or affinity of BZD binding sites on the $GABA_A$ receptor complex in the rat model. Further, Kimber *et al.* (1987) also reported that 21 day administrations of desipramine, TCP or zimelidine to rats did not significantly alter either the number or affinity of BZD binding sites in cortex. Similar data were also obtained by Lloyd and Pilc (1984). These findings are in conflict with the findings by Suranyi-Cadotte *et al.* (1985, 1990) of a decrease in the density of BZD sites following chronic administration of antidepressants. As the above studies indicate, the involvement of the BZD binding site in the mechanisms of actions of antidepressant drugs remains questionable and further research is required.

1.3.4. GABA and Panic Disorder:

Panic attacks represent acutely incapacitating episodes, often unassociated with an environmental precipitant, characterized by massive autonomic discharge, acute anxiety, and fear of impending doom (Keller and Hanks, 1993). Panic attacks may or may not be associated with agoraphobia or a generalized anxiety disorder. Panic disorder (PD) has been recognized for at least 100 years as a psychopathological condition. Over the last several decades, health professionals have changed their view of PD and in 1987 in the Third Edition (Revised) of the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-III-R) PD was included as a distinctive syndrome (Keller and Hanks, 1993)

Until recently, anxiety and depression have generally been considered to be distinct clinical dimensions, with anxiety responding to BZDs and depression to antidepressants. However, evidence from epidemiological (Stravrakaki and Vargo, 1986) and phenomenological (Goldberg *et al.*, 1987) studies indicate that the boundary between these two disorders is not so clear. There is evidence to show that 50-75% of patients with PD have also had at least one episode of major depression (Keller and Hanks, 1993). In addition to the coexistence of anxiety and depression within the same individuals and the high frequency of both disorders within the same families (Leckman *et al.,* 1985; Brawman-Mintzer *et al.,* 1993; Gulley and Nemeroff, 1993), there is now evidence that certain biochemical abnormalities may be common to both disorders and that tricyclics and MAO inhibitors are therapeutically effective in anxiety states (Suryani-Cadotte, 1990).

It has been suggested that most antidepressant and antianxiety drugs that produce antipanic effects also augment GABA transmission (Patel *et al.,* 1975; Korf and Venema, 1983; Sheenhan and Davidson, 1983; Lloyd and Pilc, 1984; Breslow *et al.,* 1989). Petty *et al.* (1990) found reduced plasma levels of GABA in patients with mood disorders and panic disorder.

In general, the benzodiazepines are considered the drugs of choice in the treatment of anxiety disorders (Hollister, 1978). Yet, despite their common use, there is a growing concern over adverse effects such as tolerance, dependence and withdrawal effects associated with long term use of benzodiazepines (Owens and Tyrer, 1983; Blais and Petit, 1990). Further, there are several forms of anxiety that do not respond to benzodiazepine treatment (Kelly, 1973). Therefore, there is considerable interest in identifying other classes of drugs that are capable of alleviating all forms of anxiety without producing tolerance or withdrawal effects.

Clinical studies have shown that MAO inhibitors and tricyclic antidepressants may actually be more effective than the benzodiazepines in alleviating the anxiety associated with panic attacks and agoraphobia (Sargant and Dally, 1962; Kelly, 1973; Ballenger *et al.*, 1977; Breslow *et al.*, 1989). In animals, acute and chronic treatment with tricyclics and MAO inhibitors has been reported to increase functional brain GABA activity, either by inhibiting reuptake or degradation, or by increasing release (Patel *et al.*, 1975; Korf and Venema, 1983; Baker *et al.*, 1992; McManus *et al.*, 1991). Therefore, the mechanism involved in the antipanic efficacy may be an increased functional availability of brain GABA.

Phenelzine (PLZ) has been shown to have beneficial effects in the treatment of PD and has even been suggested as the drug of choice in the treatment of this disorder (Ballenger, 1986; Breslow *et al.*, 1989; Sheehan *et al.*, 1990). This drug, although primarily a nonspecific inhibitor of MAO, has also been found to induce a substantial increase in the levels of GABA and another amino acid, alanine, in rat brain (Wong *et al.*, 1989; Baker *et al.*, 1991).

1.3.5. Phenelzine

PLZ is a hydrazine-containing compound structurally similar to 2-phenylethylamine (PEA) and amphetamine (see Figure 4.) and, in addition to its inhibition of MAO, has weak to moderate effects on the uptake and/or release of dopamine, NA and 5-HT (Baker *et al.*, 1978). PLZ has been used in the treatment of a variety of disorders, including major depression, atypical depression (Liebowitz *et al.*, 1985; Quitkin *et al.*, 1990); panic disorder (Sheehan *et al.*, 1980) bulimia, social phobia (Liebowitz *et al.*, 1985) and post-traumatic stress disorder (McDaniel, 1986).

Uniquely, PLZ is not only an inhibitor of MAO, but it is also metabolized by MAO (Baker and Coutts, 1989). This paradoxical situation has made delineating the metabolism of PLZ a complex undertaking. For many years, PLZ was thought to undergo acetylation, but this route of metabolism is currently a matter of debate (Robinson *et al.*, 1985; Mozayani *et al.*, 1988; McKenna *et al.*, 1991,1992). Other routes of metabolism include biotransformation to PEA and phenylacetic acid and possibly ring hydroxylation to form para-hydroxy-PLZ, para-tyramine and para-hydroxyphenylacetic acid (Baker and Coutts, 1989). PLZ is rapidly absorbed, with concentration maxima occurring between 2 and 4 h; the plasma elimination half-life has been reported to range from 1.5 to 4 h (Robinson *et al.*, 1985).

Popov and Matthies (1968) reported a dose-dependent increase of brain GABA subsequent to IP injection of PLZ. This finding was later confirmed by Baker *et al.* (1991) and it was suggested that it was this action of PLZ, that is, the elevation of GABA, that was relevant to its antipanic efficacy. Interestingly, Popov and Matthies (1968) also found that pretreatment of rats with the nonspecific MAO inhibitor TCP reversed the GABA-elevating action of PLZ. It

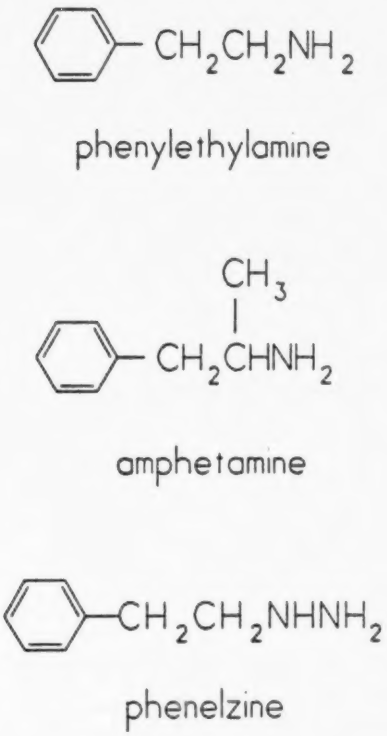

CH$_2$CH$_2$NH$_2$
phenylethylamine
CH$_3$
CH$_2$CHNH$_2$
amphetamine
CH$_2$CH$_2$NHNH$_2$
phenelzine

Figure 4. Structures of PLZ, β-PEA and amphetamine (AMPH).

was of interest then to investigate further the interactions of PLZ with the GABAergic system, and it is to this end that a portion of the present thesis is dedicated.

1.4 RECEPTOR BINDING

As much of the data previously described is based on experiments employing receptor binding techniques, and this methodology was also used in some of the experiments described in this thesis, a brief description of the methodology and parameters measured is required. In the characterization of a radioligand binding assay, the binding of the radioligand to the membrane preparation must meet certain basic criteria in order to be recognized as binding that is biologically meaningful to a specific receptor: 1) saturation and reversibility, and 2) physiological and pharmacological specificity (Hrdina, 1986).

The minimal requirement for binding to be of biological interest is that it be saturable. There is only a finite number of specific receptor sites per unit of tissue, and as the concentration of radioligand increases, these sites become fully occupied. The binding of the radioligand to "nonspecific" sites, such as filters and glassware, is not saturable within a reasonable range of radioligand concentrations. Reversibility of binding is most easily illustrated through an examination of how specific and nonspecific binding components are determined. In a typical saturation binding assay, a fixed amount of tissue is incubated with increasing concentrations of the radioligand in the presence (nonspecific binding) or absence (total binding) of an excess amount of nonradiolabelled molecules of a compound which competes for the receptor. Specific binding is then defined as the difference in radioactivity between total and nonspecific binding. Nonspecific binding can be determined by the addition of unlabelled drug because, through competition at the specific receptor, the reversibly bound radioligand is displaced by the unlabelled compound. If the radioligand is irreversibly bound, no competitive displacement of the ligand can occur. By default, the radioactive ligand not displaced must be bound to nonspecific sites. A binding curve outlining the relationship among these 3 components is shown in Figure 5.

The second basic requirement of binding site/receptor characterization is physiological or pharmacological specificity. The most important feature of pharmacological specificity is a high degree of correlation between the potencies of unlabelled drugs to displace the radioligand from the specific binding site and their potencies in producing a biological response (Burt, 1978). A strong correlation should also exist between the affinity of a drug for the specific binding site *in vitro* and its pharmacological potency *in vivo* (Hrdina, 1986).

The binding assays employed in most direct studies of ligand-receptor interaction involve incubation of a suitable tissue receptor preparation with an isotopically labeled ligand (agonist or antagonist), separation of the bound from

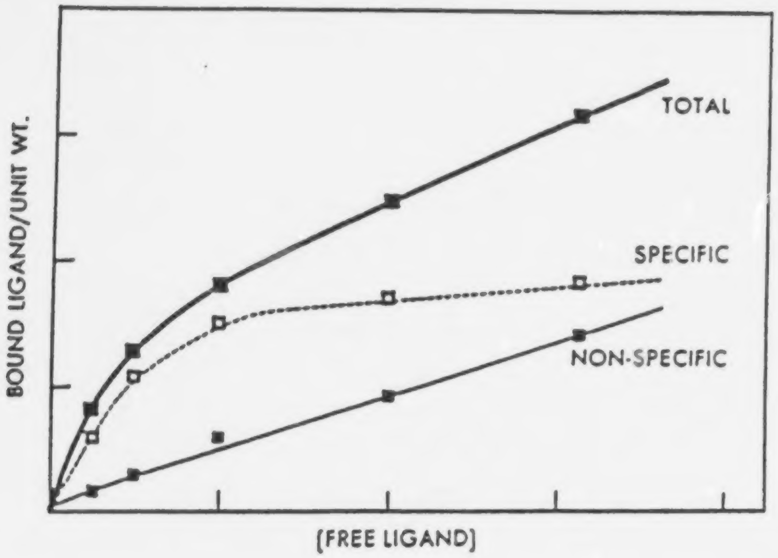


Figure 5. Typical binding curves generated from a direct binding assay. Reprinted with permission from Humana Press from *Neuromethods, Volume 4, Receptor Binding*, AA Boulton, GB Baker and P Hrdina (eds), 1986, p.8.

the free ligand, and determination of the bound radioactivity (Hrdina, 1986). The assumption underlying binding studies is that the ligand-receptor binding is a reversible bimolecular reaction that at equilibrium obeys the law of mass action and is described by the following equation:

$$[L] + [R] \leftrightarrow [LR]$$

[L] represents the concentration of the free ligand, [R] the concentration of the free receptor, and [LR] the concentration of the ligand-receptor complex. The equilibrium dissociation constant, **K_D**, for the ligand-receptor interaction is used as a measure of affinity of the ligand for the receptor and is defined by the equation:

$$K_D = [L] \times [R] / [LR]$$

A second value of interest in binding studies is the maximum number of specific binding sites - the **Bmax**. Both Bmax and K_D values are estimated from a Scatchard plot (Figure 6.) of the saturation binding data by using the Scatchard equation:

$$B/F = Bmax - B / K_D$$

In this equation, B represents the amount of bound ligand, and F the amount of unbound or free ligand.

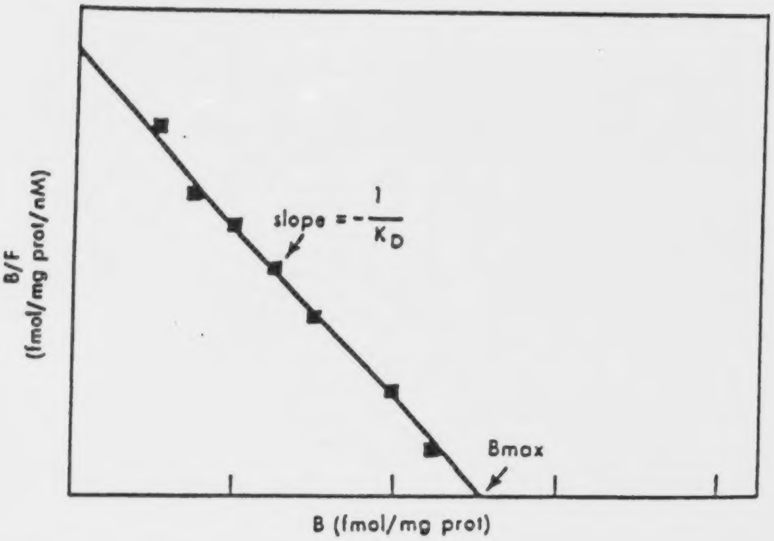


Figure 6. A typical Scatchard plot of binding data. Reprinted with permission from Humana Press from *Neuromethods, Volume 4, Receptor Binding,* AA Boulton, GB Baker and P Hrdina (eds), 1986, p.10.

1.5 HPLC and GC ANALYSIS

Several of the studies described in this thesis have utilized procedures employing high pressure liquid chromatography (HPLC) for the analysis of biogenic amines and their acid metabolites, and gas chromatography (GC) for the analysis amino acids and drugs. HPLC allows for the determination of selected biogenic amines directly in the supernatants of tissue homogenates or in CSF without prior sample purification (Warsh *et al.,* 1982). The basic mechanism of analyte separation involves the mass transfer of analyte between the mobile (liquid) and stationary phases. Transfer of the analyte is dependent on a number of factors such as the sorbent surface area and electrical charge, analyte electrical charge and polarity, and mobile phase composition and polarity. HPLC is generally classified into normal phase, ion-exchange and reversed-phase chromatographies, depending on whether the principal physical processes underlying analyte separation involve adsorption, ionic affinity or phase partition (Warsh *et al.,* 1982). Further, the ultimate sensitivity and specificity of HPLC procedures for the biogenic amine determination depend upon the characteristics and mode of application of the detection system used. Currently, there are various detector systems available, including optical systems such as ultraviolet absorbance, fluorescence and refractive index detection, electrochemical detectors and vapor phase detectors such as thermo-conductivity, flame ionization, electron capture and mass spectometric detector systems (Warsh *et al.,* 1982).

Currently, because of its suitability, the electrochemical detector is the most popular detector type for the analysis of catecholamines, indoleamines and their acid metabolites and hence is the one utilized for experiments included in this thesis. Compounds with phenolic groups or the indole structure are easily oxidized and this reaction can be electrochemically induced as the process only requires electron transfer. Electrochemical procedures are based on the measurement of a current, either generated or consumed, associated with a redox reaction of an electrochemically active analyte on the surface of an electrode. The change in current is proportional to the concentration of analyte causing these changes. Quantitative electrochemical detection is possible if ionic strength, pH and temperature of the mobile phase are kept constant. Additionally, the electrochemical detector can be selective, as optimum oxidation potentials used to oxidize different compounds are different. Therefore, with proper selection of oxidation potential, certain compounds can be oxidized while excluding others.

Commercially available electrochemical detectors can be divided into two categories: amperometric and coulometric (review: Scott, 1986). The basic difference between these two detectors is the relative size of their cell surface area and thus their electrolytic efficiency ranges. Amperometric detectors have a smaller cell surface area and an electrolytic efficiency range of only 1-10% of maximum. Coulometric detectors on the other hand have a larger electrode surface area and an electrolytic efficiency approaching 100%. The smaller cell surface area provides low dead volumes, low background current and small flow fluctuations. With the larger cell surface areas in the coulometric detectors, larger dead volumes and greater baseline noise result.

After separation in the analytical column, electrochemically-active analytes elute from the column and enter the electrochemical cell where they are oxidized (or reduced) at the working electrode surface and removed after contact with reference and auxiliary electrodes. Glassy carbon is the material of choice for the working electrode; silver/silver chloride electrodes and stainless steel or platinum electrodes are widely used as reference and auxiliary electrodes respectively.

GC is also an analytical procedure that is used to separate mixtures of organic compounds for the purposes of identification and/or quantification. In contrast to HPLC which allows for the determination of analytes directly from tissue homogenates, GC separations are usually performed on solutions of compounds in inert solvents (Baker *et al.,* 1982). Components of a mixture in solution are initially vaporized and carried through a column (contained in the oven of the GC) by an inert carrier gas. The components separate from one another according to their partition coefficients between the carrier gas and the stationary phase of the column (Baker *et al.,* 1982). As with HPLC, there are a number of detector types available for GC analysis: thermal conductivity detectors, flame ionization detectors, electron-capture detectors, nitrogen-phosphorous detectors and mass spectrometric detection.

In this thesis, both the nitrogen-phosphorus (NPD) and electron-capture detectors (ECD) were utilized. The NPD is a relatively sensitive detector and is extremely sensitive to most compounds which possess nitrogen and/or phosphorus-containing functions (Baker *et al.*, 1982). The operation involves mixing the effluent from the GC column with a smaller volume of hydrogen; this mixture then enters an electrically heated detector chamber which contains a rubidium salt. A low-temperature plasma is formed and produces a minute electric current of a magnitude which is proportional to the amount of compound reaching the detector. The current is then amplified and recorded. The sensitivity for the detection of nitrogen- and phosphorous-containing compounds is in the low picogram range (Baker *et al.*, 1982)

The ECD is also relatively selective and has the potential to detect as little as 1 picogram of an organic compound containing an electrophoric substituent. In its operation, the radioactive source (^{63}NI) emits relatively high energy β-particles. The particles collide with carrier gas molecules and produce a small current (the standing current). When an electrophoric compound elutes from the GC column, it captures electrons and thus reduces the strength of the standing current (Lovecock and Lipsky, 1960; Sevcik, 1976), which returns to its original level when the sample has left the detector. The change in current is amplified and appears as a peak on the recorder.

1.6. THESIS OBJECTIVES

The initial objective of the thesis was to compare the effects of chronic administration of three classes of antidepressants (tricyclics, MAO inhibitors and novel) on the BZD and 5-HT$_2$ receptors in rat brain. The focus then moved on to further clarify the mechanisms of action of PLZ on brain GABA levels. A study was conducted to determine whether inhibition of MAO-A, MAO-B or both was relevant to the elevation of GABA levels in brain by PLZ. A comparison was made with two other known hydrazine-containing MAO inhibitors on GABAergic mechanisms in rat brain. PLZ was also compared to a specific GABA-T inhibitor (vigabatrin) with regard to the ability to elevate brain GABA and ALA. These studies are presented in separate chapters to permit a brief but specific introduction relevant to each.

1.7. GENERAL METHODS

1.7.1. Chemicals Used

Table 1. Chemicals used in the experiments described in this thesis.

Chemicals	**Suppliers**
acetic acid - glacial	BDH Chemicals (Toronto, Ont)
acetic anhydride	Caledon Laboratories (Georgetown, Ont)
acetonitrile, HPLC grade distilled in glass	BDH Chemicals
^{3}H-alanine	Dupont, NEN Products (Boston, MA)
γ-aminobutyric acid	Aldrich (Milwaukee, WI)
^{3}H-γ-aminobutyric acid	Dupont, NEN Products
2-aminoethylisothioluronium bromide	Sigma
ascorbic acid	Fisher Scientific
bovine serum albumin	Sigma
chloroform, reagent grade	Fisher Scientific
clorgyline	Research Biochemicals Inc. (Wayland, MA)

clomipramine HCl	CIBA-GEIGY Corp. (Summit, NJ)
D,L-isoleucine	Sigma
D.L-norleucine	Aldrich
D,L-valine	Nurtitional Biochemical Corp. (Cleveland, OH)
(-)-deprenyl HCl	Research Biochemicals Inc.
deoxycholate	Fisher Scientific
dopamine HCl	Sigma
desmethylimipramine	Sigma
dicyclohexylcarbodiimide	Aldrich
diethyl ether	BDH Chemicals
dithiothreitol	Sigma
3,4-dihydroxyphenylacetic acid	Sigma
ethyl acetate	BDH Chemicals
(±)-fluoxetine	Sigma
folin - phenol reagent	Sigma

^{14}C-glutamic acid	Amersham Canada Ltd (Oakville, ON)
glutathione	Sigma
glycerol	Sigma
homovanillic acid	Sigma
hydrochloric acid	Fisher Scientific
5-hydroxyindole-3-acetic acid	Sigma
^{14}C-5-hydroxytryptamine	Dupont, NEN Products
5-hydroxytryptamine creatine sulfate	Sigma
iproniazid	Sigma
isopentane	BDH Chemicals
isobutyl chloroformate	Aldrich
α-ketoglutarate	Sigma
^{3}H-ketanserin	Dupont, NEN Products
L-alanine	Aldrich
L-glycine	Sigma
L-leucine	Sigma

maprotiline	Ciba-Geigy
mianserin	Sigma
nialamide	Sigma
nicotinamide adenosine dinucleotide	Sigma
(-)-noradrenaline HCl	Sigma
pentafluorophenol	Aldrich
perchloric acid	Fisher Scientific
phenelzine sulfate	Sigma
β-phenylethylamine HCl	Sigma
^{14}C-β-phenylethylamine	Dupont, NEN Products
phosphoric acid	Fisher Scientific
potassium carbonate anhydrous	Fisher Scientific
potassium chloride	Fisher Scientific
pyridoxal phosphate	Sigma
scintillation fluid (Ready Safe™)	Beckman Instruments Inc. (Edmonton, AB)
sodium bicarbonate	Fisher Scientific

sodium carbonate anhydrous	Fisher Scientific
sodium chloride	Fisher Scientific
sodium hydroxide	Fisher Scientific
sodium phosphate, dibasic, anhydrous	Fisher Scientific
sodium phosphate monobasic	Fisher Scientific
sodium potassium titrate	Allen & Hanbury's (Toronto, ON)
sucrose	Fisher Scientific
toluene, distilled in glass	BDH Chemicals
toluene, reagent grade	BDH Chemicals
(±)-tranylcypromine	Sigma
tri-n-octylamine	Sigma
tris(hydroxymethyl)aminomethane (TRIS)	Fisher Scientific
Triton x-100	Terochem Lab. Ltd. (Edmonton, AB)
vigabatrin	Marion Merrell-Dow (Richmond Hill, ON)

1.7.2. INSTRUMENTATION AND APPARATUS

1.7.3. *Filtration*

A Brandel Cell harvester equipped with Whatman GF/C filters was used for the filtration steps in all receptor binding assays. This harvester allowed for the simultaneous filtration of 48 samples.

1.7.4. *Centrifuges*

A Sorvall GLC-2B or Sorvall GLC-1 General Laboratory Centrifuge (Dupont Instruments) was used for low-speed, small volume centrifugations. Higher speed and/or larger volume centrifugations were carried out in a Damon-IEC B-20 refrigerated high-speed centrifuge or a Beckman L755 vacuum refrigerated ultracentrifuge.

1.7.5. *Gas Chromatography*

For determination of the tricyclic antidepressants a Hewlett Packard (HP) Model 5890 gas chromatograph equipped with a fused silica column and a nitrogen phosphorus detector linked to an HP 3392A integrator was used. The carrier gas was pure helium at a flow rate of 3.0 ml/min. The detector was purged with pure hydrogen (3.5 ml/min) mixed with dry air at 80 ml/min. Injection port and detector temperatures were 200°C and 325°C respectively.

Determination of amino acid levels was carried performed on an HP 5890 gas chromatograph equipped with a fused silica column, an electron-capture detector (ECD) with a radioactive source of 15 mCi Nickel-63, an HP 7673A automatic sampler and an HP 3392A integrator. The carrier gas, helium, was set at a flow rate of 2 ml/min. Argon-methane (95%-5%), flow rate 35 ml/min, was the make-up gas used in the detector. The injection port temperature was 200°C and the detector temperature was 325°C.

1.7.6. *High pressure liquid chromatography (HPLC)*

Levels of the biogenic amines and their acid metabolites were determined using an HPLC system consisting of a solvent delivery system (Model 510, Waters Associates, Milford, MA) coupled to an automatic injector (WISP, Waters model 710B). The compounds of interest were separated on an Econosphere™ C_{18} column (4.6 mm i.d. x 250 mm, 5μm particle size, Applied Science Labs, Avondale, PA). A precolumn (4.6 mm i.d. x 30 mm) with the same packing material as that in the analytical column was used. Elutants from the column were detected by an electrochemical detector (model 460, Waters) with the applied potential set at 0.90 volt. Chromatographic peaks were recorded and integrated using a model 740 integrator (Waters). The mobile phase, pumped at a flow rate of 0.7 ml/min, consisted of 55 mM sodium phosphate monobasic, 0.73 mM sodium octyl sulfate, 0.37 mM disodium EDTA and 6.5% v/v acetonitrile; the pH was adjusted to a value of 2.75 with phosphoric acid.

1.7.7. *Liquid Scintillation Spectrometry*

A Beckman LS 7500 liquid scintillation spectrometer coupled to a Datamex 43 printer was used for counting radioactivity in all receptor binding assays, and in procedures for analysis of activities of MAO, GABA-T and ALA-T.

1.7.8. *Ultraviolet Spectrophotometer*

A Pye Unicam SP 1700 ultraviolet spectrophotometer was used for determination of protein concentrations in homogenates used in receptor binding studies.

1.7.9. *Tissue Homogenizer*

A combination of a TRI-R S63C variable speed laboratory motor with a Teflon™ glass pestle and a glass grinding tube was used for homogenizing all tissue samples. For the sake of consistency, a setting of 7 was used at all times.

1.7.10. *Shaker-Mixer*

Two types of vortex-shakers were used: Ika-Vibrax VXR2 Shaker™ (Janke and Kunkel Instruments) and a Thermolyne Maxi Mix™ vortex mixer (Sybron/Thermolyne Instruments).

1.7.11 *Weighing Balances*

A Mettler AE 160 electronic balance was used for weighing chemicals and biological samples.

1.7.12. *Glass Cleaning*

All glassware was rinsed with tap water and washed with biodegradable Sparkleen™ (Fisher Scientific Co.) solution. Further washing was accomplished with a dishwasher (Miele Electronic 6715). For test tubes, an additional step was added: test tubes were sonicated (ultra-sonic cleaner, Mettler Electronics) in a solution of Decon 75 concentrate (BDH Chemicals) before the dishwasher wash. After removal from the dishwasher, all glassware was air-dried in a mechanical convection oven Model 28, Precision Scientific Group.

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CHAPTER 2

Chronic administration of phenelzine, desipramine, clomipramine or maprotiline decreases 5-HT$_2$ receptor binding without affecting benzodiazepine binding sites in rat brain.

(The work presented in this chapter formed the basis for two submitted manuscripts, one on the development of an assay procedure for clomipramine and one on the effects of several antidepressants on 5-HT$_2$ and benzodiazepine binding sites in rat brain.)

2.1 INTRODUCTION

Ever since the introduction of the original theories suggesting that depression was the result of a functional deficiency of biogenic amines at central synapses (Bunney and Davis, 1965; Schildkraut, 1965; Lapin and Oxenkrug, 1969) research on depressive disorders has been concentrated on a search for biochemical lesions involving the amines NA, 5-HT and, to a lesser extent, dopamine (DA) (Baker and Dewhurst, 1985). In recent years, there has been increased interest in the possible role of the neurotransmitter amino acid GABA in the etiology and pharmacotherapy of affective disorders (Lloyd *et al.*, 1989; Breslow *et al.*, 1989; Suranyi-Cadotte *et al.*, 1985).

Literature reports regarding the role of the GABAergic systems in depressive disorder are quite varied. Injections of GABA into various brain regions have been shown to prevent "learned helplessness" behavior, which is considered by some to be an animal model of human depression (Sherman and Petty, 1982). Tricyclic antidepressants desipramine (DMI), imipramine and trimipramine were shown to increase the release of endogenous GABA from rat thalamus (Korf and Venema, 1983). Further, chronic administration of electroconvulsive shock to rats has been reported to increase concentrations of and decrease the synthesis of GABA in the nucleus accumbens and caudate nucleus (Green *et al.*, 1978).

Clinical studies have reported reduced cerebral spinal fluid levels of GABA in depressed patients (Gold *et al.*, 1980). Perry *et al.* (1977) reported a decrease in GAD activity in several brain regions of depressed patients, but these findings have not been replicated. Cheetham *et al.* (1988) reported an increased number of benzodiazepine (BZD) binding sites in the frontal cortex of depressed suicide victims. This is of particular interest since recent molecular cloning experiments have shown conclusively that the benzodiazepine binding sites are located on the $GABA_A$ receptor complex (Pritchett *et al.,* 1989).

In terms of GABA receptor findings, chronic administration of antidepressants of every class (tricyclics, MAO inhibitors and novel) as well as repeated electroshocks have been reported to result in an up-regulation of $GABA_B$ receptors in rat cortex (Lloyd *et al.,* 1985; Suzdak and Gianutsos, 1986). These findings are somewhat controversial, however, as other laboratories, including our own, have found no changes in $GABA_B$ receptor density following chronic antidepressant treatment (Cross and Horton, 1987; McManus and Greenshaw, 1991). A chronic study investigating the effects of a variety of antidepressants on BZD binding sites found a significant decrease in the number of these sites in rat brain (Suryani-Cadotte, 1985). This finding, too, remains controversial, as a more recent study found no change in BZD binding site number in rat brain following chronic administration of desipramine (DMI) or tranylcypromine (TCP) (Kimber *et al.,* 1987).

As the above brief review of the literature indicates, there remains a great deal of uncertainty regarding the role of the GABAergic system and its related binding sites in depressive disorder. To aid in clarifying the role of the

$GABA_A$ receptor complex in depression, the BZD binding site requires further investigation. To this end, the effects of chronic (21 day) treatment of several different types of antidepressants on BZD binding sites in rat cortex were investigated. Chemical structures of the drugs evaluated in this experiment are shown in Figure 7. As recent reports have indicated that a down-regulation of 5-HT_2 receptors after chronic drug administration is a property shared by several antidepressants (Eison *et al.*, 1991; Lafaille *et al.*, 1991), the effects of the drugs on 5-HT_2 receptors in the hippocampus of the same experimental animals were assessed.

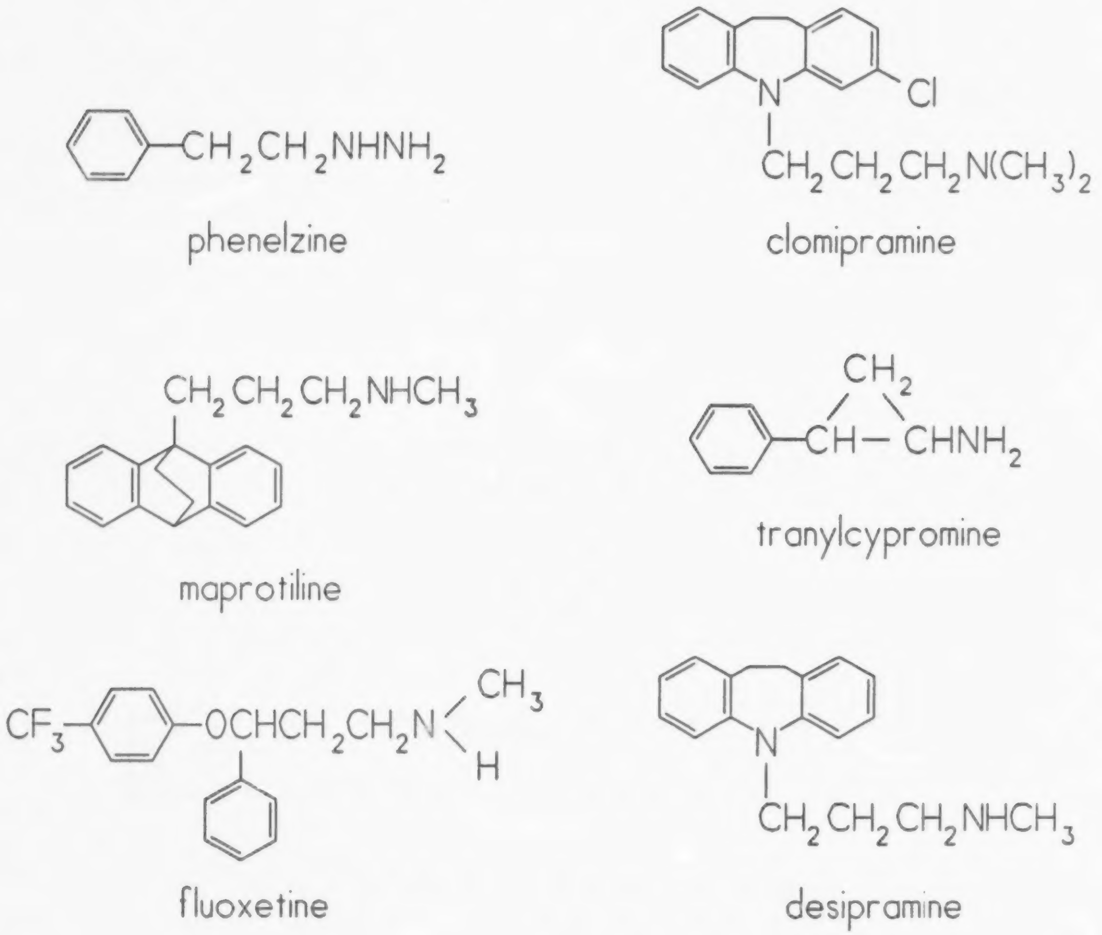
2.2. METHODS

2.2.1 Animals:

Male Sprague-Dawley rats weighing 250-300g at the time of surgery were used. Animals were housed in pairs with free access to food and water on a 12 h light:dark cycle with ambient temperature maintained at 21 ± 1°C.

2.2.2 Drug administration:

Rats were randomly assigned to groups (n=10) receiving one of the following drugs: phenelzine (PLZ), DMI, fluoxetine (FLUOX), clomipramine (CMI), maprotiline (MAP) (all at a dose of 10 mg/kg/day based on free base weight) or the appropriate vehicle. These drug doses were chosen based on previous results in our laboratory and on their common use in other published reports in



phenelzine

clomipramine

maprotiline

tranylcypromine

fluoxetine

desipramine

Figure 7. Chemical structures of the drugs used in the present study.

the literature. Animals were anesthetized with the inhalant anaesthetic methoxyflurane and a 2ML4 Alzet osmotic minipump implanted subcutaneously in the interscapular region. This surgical procedure consisted of a small incision and the creation of a pocket by separating the overlying skin from the muscle facia. The pump was then inserted into the pocket, moderator end first, the incision closed with wound clips, and an antibiotic powder applied to prevent infection. After 21 days of continuous drug administration, the animals were sacrificed by guillotine decapitation and the brains dissected for the cortex (whole) and hippocampus. To obtain the hippocampi, the brain was initially halved along the midline, and placed on the dorsal aspect. The midbrain tissue was removed with microdissection tweezers. The hippocampi were removed by unfolding the caudal and lateral edges of the cortex and clipping off the left and right hippocampi. The cortex was then cleaned of all remaining tissue. These regions and the rest of brain were frozen immediately and stored at -80°C until analysis.

2.2.3 MAO Activity Analysis:

Brain MAO activity was measured in rest of brain by a modified version of Wurtman and Axelrod's radiochemical method (1963) using ^{14}C-5-HT and ^{14}C-β-phenylethylamine (PEA) as substrates for MAO-A and MAO-B respectively. Tissue for this assay was homogenized in 5 vols of distilled H_20 and aliquots of 200 μl were diluted to 1000 μl with ice-cold isotonic KCl. Sodium phosphate buffer (250 μl, pH 7.4) and 25 μl of tissue homogenate (or 25 μl of KCl in the blanks) were added to tubes kept on ice. Aliquots (25 μl) of [^{14}C]-5HT or [^{14}C]-PEA, diluted with the respective unlabeled compounds, were added to each tube to give a final concentration of 100 and 10 μM, respectively of substrate in each. The tubes were then incubated at 37°C for 20 min. The enzyme reaction was terminated with the addition of 200 μl of 2 N HCl. The metabolites formed in the reaction were extracted into 6 ml of toluene by mixing for 3 min and centrifuging at 1000 x g for 5 min. Following extraction, the tubes were kept at -80°C for not less than 1 h to freeze the aqueous layer. The toluene layer (top) was transferred to scintillation vials and 9 ml of scintillation cocktail was added to each. Radioactive content per tube was determined by using a liquid scintillation spectophotometer. To ascertain percent inhibition first, the amount of radioactivity in the blank tubes was subtracted from all samples and controls. Then, the following formula was applied to the values to obtain percent inhibition:

% inhibition = 100-[100x((sample-blank)/(control-blank))]

2.2.4 Drug Level Analysis:

Brain levels of DMI, FLUOX, CMI, and MAP were determined using gas chromatographic assays. Specifically, for DMI, CMI and MAP rat brain tissue was homogenized in 5 volumes of ice-cold distilled water and a 2 ml portion of the homogenate was used for anaylsis. The internal standard (MAP for DMI and CMI, and DMI for MAP) at a concentration of 1000 ng was added to the homogenate which was basified with solid sodium bicarbonate. This mixture was then extracted with ethyl acetate, centrifuged and the ethyl acetate layer dried under a stream of nitrogen gas. The remaining residue was reconstituted in 2 ml of double distilled H_20. Acetylation was carried out by adding acetic anhydride in basic conditions as described by Drebit *et al.* (1988). The acetylated compounds and underivatized CMI were extracted by shaking with ethyl acetate for 10 min. After a brief centrifugation, the organic phase was transferred to another set of tubes and evaporated to dryness under a stream of nitrogen gas. The samples were redissolved in toluene and aliquots of these solutions were injected onto a gas chromatograph equipped with a fused silica capillary column (25m x 0.32mm I.D.x 1.05 μm film thickness, cross-linked 5% phenylmethylsilicone phase), a nitrogen-phosphorus detector and a printer-integrator. Separation of the compounds of interest was accomplished using the following automatic oven temperature program: initial temperature of 105°C for 0.5 min, increasing at a rate of 25°C/min to 295°C where it was held for 5 min.

To detect the presence of FLUOX in rat brain, the tissue was homogenized in 5 volumes of ice-cold distilled water and a 2 ml portion of the homogenate was used for analysis. The internal standard p-chlorophentermine at a concentration of 1000 ng was added to the homogenate which was then basified with 25 % potassium carbonate. This mixture was extracted with the organic solvent toluene. The organic layer (top) was removed and taken to dryness under a stream of nitrogen gas. The residue was reconstituted in toluene and derivitized with the agent pentafluorobenzoyl chloride (PFBC) for 30 min at 60°C as described by Aspeslet *et al.* (1993). The mixture was allowed to cool to room temperature and was then washed with sodium borate. The toluene layer was removed for injection onto a gas chromatograph equipped with a fused silica capillary column (25m x 0.32 mm I.D.), cross-linked 5% phenylmethylsilicone phase (1.05 μm film thickness), electron-capture detector and a printer-integrator. Separation of the compounds of interest was accomplished using the following automatic oven temperature program: initial temperature or 105°C for for 0.5 min, increase at a rate of 25°C/min to 295°C where it was held for 5 min. As with all GC assays, a standard curve was run concomitantly with the tissue samples. This curve was constructed by adding known, varying amounts of authentic standards of the drug of interest and a fixed amount (the same amount as added to the tissue samples) of internal standard to a series of tubes containing drug-naive tissue and carrying these tubes through the assay procedure in parallel with the sample tubes. Upon completion of GC analysis, a regression analysis was performed on the standard curve to check for linearity. Typically, correlation coefficients of $r > 0.99$ were achieved. Peak heights of the derivatized drug of interest to derivatized internal standard were plotted against known concentrations of the drug of interest, and quantification of drug levels ascertained by plotting the peak height ratios from the brain samples on the same curve.

2.2.5 Clomipramine Assay:

MAP amd DMI had previously been assayed using the procedure described in section 2.2.4 (Drebit *et al.*, 1988; McManus *et al.*, 1990), but CMI had not. Thus, experiments to verify the percent recovery and reliability for CMI were carried out. To each test tube, in a set of 14, known amounts of CMI (250 ng), the demethylated metabolite, N-desmethylchlomipramine (NDCMI) (150 ng), and the internal standard MAP (1000 ng) were added. Seven tubes (Series A)were carried through the entire assay procedure, while the remaining seven were included at the acetylation step (Series B). Percent recovery was determined by employing the following equation:

% recovery = 100-(mean of Series B/mean of Series A)x100)

Interassay reliability of the assay for measuring CMI and NDCMI was assessed by determining coefficient of variation (cv) using a concentration of CMI of 250 ng. The coefficient was obtained by following equation:

cv = peak height/standard deviation of the peak heights x 100%

2.2.6 Receptor Binding Assays:

For the receptor binding anaylsis, saturation curves with ^3H-flunitrazepam (specific activity of 81.0 Ci/mmol) as the ligand were carried out to determine the equilibrium dissociation constant (Kd) and maximum number of benzodiazepine binding sites (Bmax) in cortex. The procedure is modified from Kimber *et al.* (1987). Membrane preparation for this assay involved homogenizing the cortex in 15 volumes of 0.32 M ice-cold sucrose. The tissue was then centrifuged for 15 min at 1000 x g and the supernatant collected. The supernatant was subsequently hand homogenized with 30 volumes of TRIS buffer (50 mM, pH 7.1). This mixture was centrifuged at 20,000 x g for 20 min at 4°C. The resultant pellet was collected and 4 washes in 30 volumes of TRIS buffer were carrried out, each time collecting the pellet, resuspending by hand and centrifuging for 25 min at 25,000 x g. A 100 μl aliquot of a final suspension of 60 volumes was used in the assay. The assay was performed on ice and a 5-point saturation curve including concentrations from 0.25 nM to 4.0 nM was performed for each rat cortex. The assay was performed in triplicate, and each tube contained the appropriate amount of buffer, radioligand, cold competitor (clonazepam) and tissue. The final incubation volume of 1 ml was kept on ice for 60 min. Specific binding was defined as the binding displaced by 1 μM clonazepam. Membrane-bound radioactivity was recovered by filtration under vacuum through Whatman GF/B filters using a Brandell cell harvester. Filters were washed with 10 ml ice-cold buffer and radioactivity determined by liquid scintillation counting at an efficiency of 70%. An outline of the [^{3}H]-flunitrazepam binding parameters is shown in Table 2. In Figure 8 is displayed a 10 point saturation curve, ranging from 0.25 nM to 12.5 nM, from which the 5 points used in the assay were chosen.

Single point analysis of 5-HT$_2$ binding sites in hippocampus was conducted with ^{3}H-ketanserin (specific activity of 60.0 Ci/mmol, 0.5nM) as the ligand (Eison *et al.*, 1991). A membrane preparation was made by homogenizing the tissue in 10 volumes of ice-cold TRIS buffer (50 mM, pH 7.5) and centrifuging at 22,000 g for 17 min. The pellet was collected and resuspended in 10 volumes of buffer. This washing was repeated a second time and a final suspension in 10 volumes of TRIS obtained. Whatman filters were presoaked for at least 3 h in 2 g/L polyethyleneimine. In triplicate, test tubes containing TRIS buffer, ^{3}H-ketanserin, mianserin (10 μM, cold competitor) and tissue for a final volume of 1 ml were incubated at 37°C for 15 min. Rapid filtration with ice-cold TRIS was undertaken, and the filters dried and deposited in scintillation vials containing 5 ml scintillation cocktail. The vials were kept at room temperature for 12 h, vortexed and placed in the scintillation counter for 5 min. An outline of the ^{3}H-ketanserin binding parameters is shown in Table 3. An 8 point saturation curve for determining (in control hippocampal membrane preparations) the appropriate nM concentration to be used in performing the single point binding is shown in Figure 9.

Tubes	Buffer (μl)	Cold ligand (μl)	Hot ligand (μl)	Tissue (μl)	nM
1-3/4-6	875/775	0/100	25	100	0.25
10-12/13-15	850/750	0/100	50	100	0.5
19-21/22-24	800/700	0/100	100	100	1.0
28-30/31-33	700/600	0/100	200	100	2.0
37-39/40-42	500/400	0/100	400	100	4.0

Table 2. Assay conditions used in the determination of ^{3}H-flunitrazepam binding to the benzodiazepine binding site.

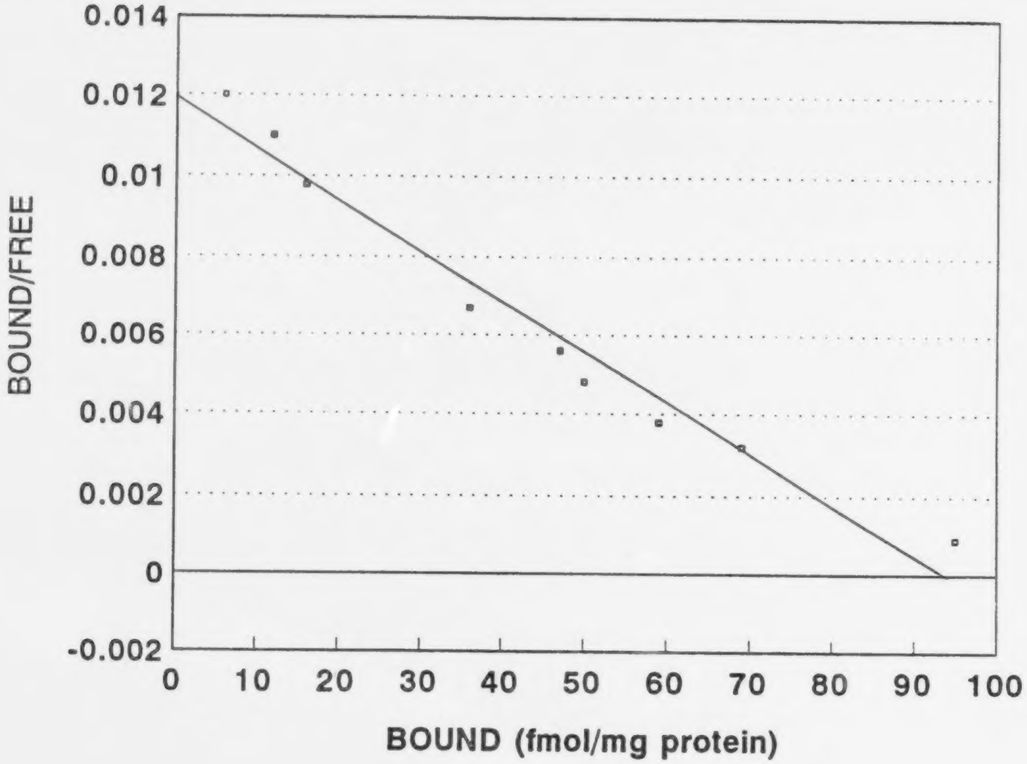


Figure 8. Full 10 point saturation curve for ^{3}H-flunitrazepam binding assay.

Tubes	Buffer (μl)	Cold ligand (μl)	Hot ligand (μl)	Tissue (μl)	nM
1-3	400	0	50	50	0.5
4-6	350	50	50	50	0.5

Table 3: Assay conditions used in the determination of ^{3}H-ketanserin binding to the 5-HT$_2$ binding site.

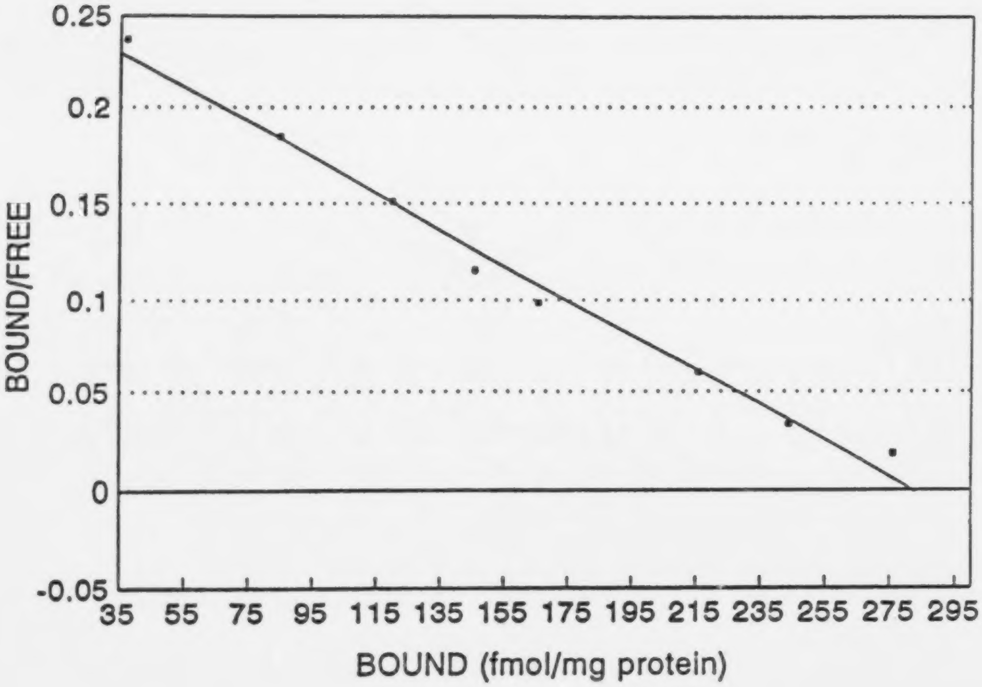


Figure 9. Full 8 point saturation curve for ^{3}H-ketanserin binding assay.

2.2.7 Protein Determination:

Protein content in rat brain homogenates was determined with Lowry's Folin Reagent assay (Lowry *et al.*, 1951). To an aliquot of 50 μl of brain homogenate were added 750 μl distilled water and 200 μl of membrane digestor (1:1 v/v 1 N sodium carbonate, 1% sodium deoxycholate). The mixture was vortexed and incubated at room temperature for 10 min. Next, 5 ml of reagent A (1/.01/.01 v/v/v 2% sodium carbonate, 1% cupric sulfate and 2% sodium potassium tartrate) were added, and the tubes were vortexed and incubated for another 10 min. Finally, 500 μl Folin reagent (1:1 v/v 2 N Folin and distilled water) was added and the tubes were vortexed and incubated for a minimum of 30 min. A standard curve was run in parallel with the tissue samples, using bovine serum albumin as protein standard. An ultraviolet/visible spectrophotometer (wave length = 660 nm) was used to determine protein concentrations.

2.2.8 Statistical Analysis:

The Bmax and K_D values were determined by linear regression analysis of Scatchard plots. To ascertain statistical significance, one way analysis of variance was applied to the data to test for main effects. Where significance was reached, *post-hoc* multiple comparisons were made between groups using Newman-Keuls comparisons. The probability level of 0.05 was chosen as indicative of significant findings.

2.3 RESULTS

To verify that the osmotic mimipumps were actually delivering the drugs to the brain, the levels of DMI, FLUOX, CMI and MAP were ascertained in the rest of brain tissue (whole brain minus cortex and hippocampus). In Figure 10 the results for the various drug groups are shown. These data confirm that the osmotic minipumps were effective in delivering the respective drugs. The drug levels obtained were similar to those reported by other researchers using similar doses of the drugs administered by osmotic minipumps (Allison *et al.*, 1993; Goodnough and Baker, 1993). The results also indicate that the acetylation procedure is an effective means of assaying for CMI and separating it from its N-demethylated metabolite (CMI is underivatized while the metabolite is acetylated).

The mean percent recoveries (n=7) obtained for 250 ng samples of CMI and 150 ng samples of NDCMI were 90.7% and 93.4% respectively. The mean interassay coeffients of variation for 250 ng samples of CMI and 150 ng samples of NDCMI were 4.3% and 7.2% respectively (n=7). Sample traces of CMI (a), MAP (b) and NDCMI (c) in derivatized extracts of brain tissue are displayed in Figure 11. Retention times (in min) were CMI, 8.80; N-acetyl-MAP, 9.84; and N-acetyl-NDCMI, 10.38.

Delivery of PLZ was verified by assaying for MAO activity in the rest of

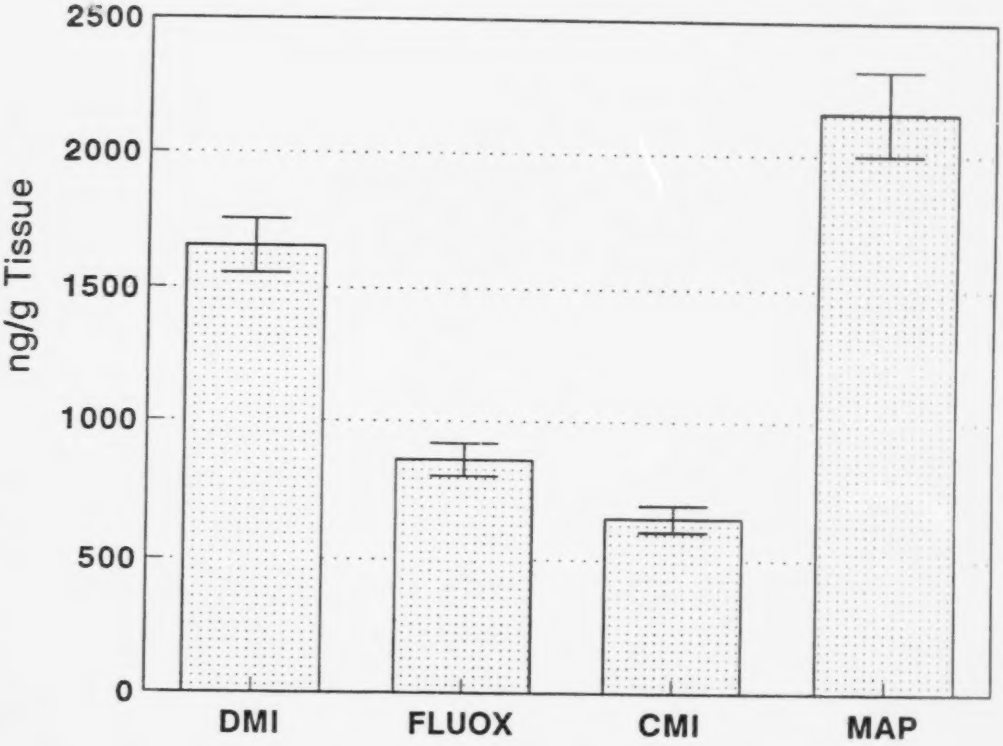


Figure 10. Drug levels (ng/g ± SEM, n=10) in whole brain (minus cortex and hippocampus) following continuous infusion of DMI (10 mg/kg/day), FLUOX (10 mg/kg/day), CMI (10 mg/kg/day) and MAP (10 mg/kg/day) *via* osmotic minipumps for 21 days.

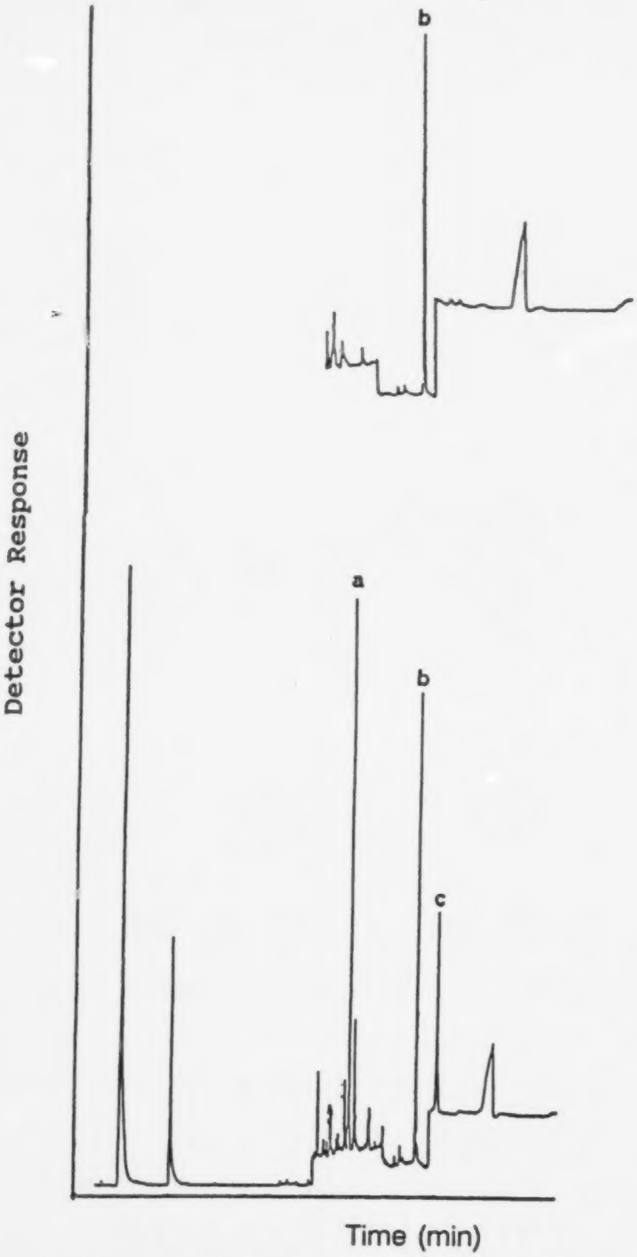


Figure 11. Sample GC traces from CMI assay in rat brain. Top: trace from an extract of control brain (vehicle administered to rats for 21 days) to which internal standard had been added): b=N-acetyl-MAP. Bottom: trace from an extract of brain from a rat treated with 10 mg/kg CMI IP for 21 days. a=CMI, b=N-acetyl-MAP, c=N-acetyl-NDCMI.

brain tissue from animals receiving this drug. The data showed that PLZ treatment resulted in highly significant inhibition (>90%) of both MAO-A and-B activity (Figure 12.).

Results from the ^{3}H-flunitrazepam binding in cortex showed that no drug tested produced significant changes in the density of benzodiazepine binding sites ($p>0.05$). These data are represented in Figure 13. In addition, there was no significant difference in K_D from control values (mean $K_D = 2.56$ nM) produced by any of the drugs. The results of the single point 5-HT_2 binding shown in Figure 14 indicate that continuous treatment with PLZ, DMI, CMI and MAP significantly decreased the binding of ^{3}H-ketanserin compared to vehicle controls ($p<0.05$). FLUOX treatment did not result in significant changes compared to controls.

2.4 DISCUSSION

No drug in these investigations produced a decrease in the Bmax or K_D of ^{3}H-flunitrazepam binding. These findings are similar to those of Kimber *et al.* (1987), who found no change in ^{3}H-flunitrazepam binding subsequent to treatment with DMI or TCP. Our results do not, however, agree with those of Suryani-Cadotte *et al.* (1985), who reported a significant decrease in ^{3}H-flunitrazepam binding following chronic administration (21 day) of a variety of antidepressants, including DMI. The different schedules of drug administration may in part explain these differences. Specifically, we employed osmotic minipumps which infuse the drugs continuously, while Suryani-Cadotte *et al.* administered the drugs by daily IP injection. As shown in this report, the administration of the drugs by osmotic minipumps resulted in effective delivery of the drugs (as monitored by measuring drug levels or MAO activity) to brain. Suranyi-Cadotte *et al.* (1985) did not report brain levels of drugs or the degree of inhibition of MAO in their study.

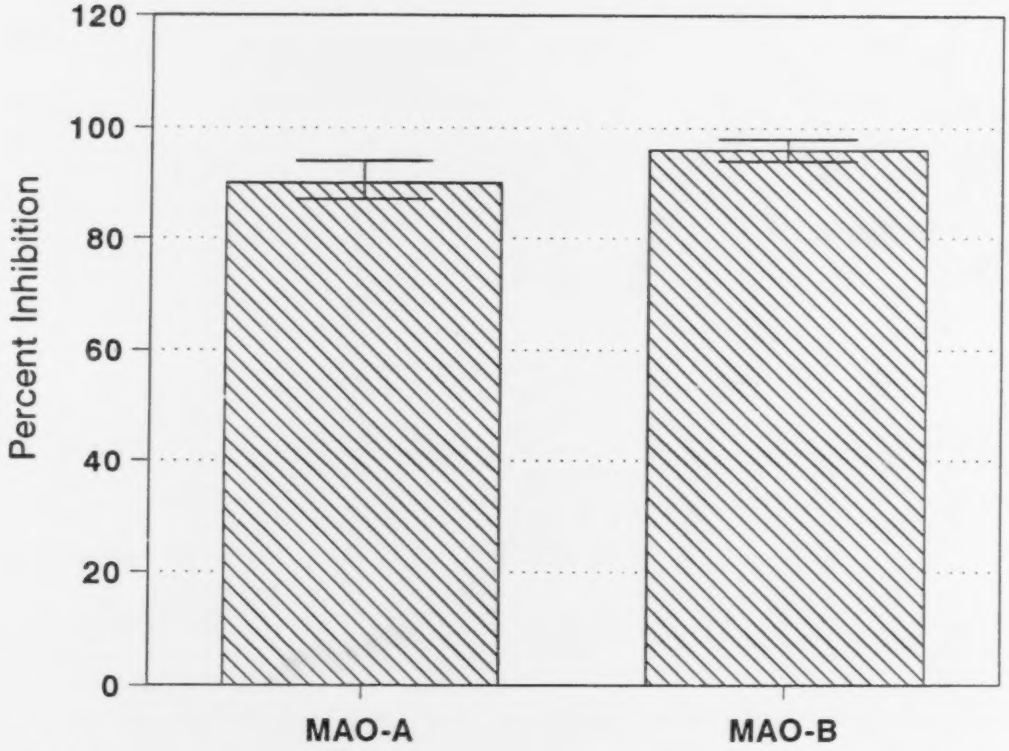


Figure 12. Percent inhibition of MAO-A and -B activity produced by administration of PLZ (10 mg/kg/day) for 21 days *via* osmotic minipumps. Results represent mean percent (± SEM; n=10) of values in brains of rats treated with VEH for the same number of days.

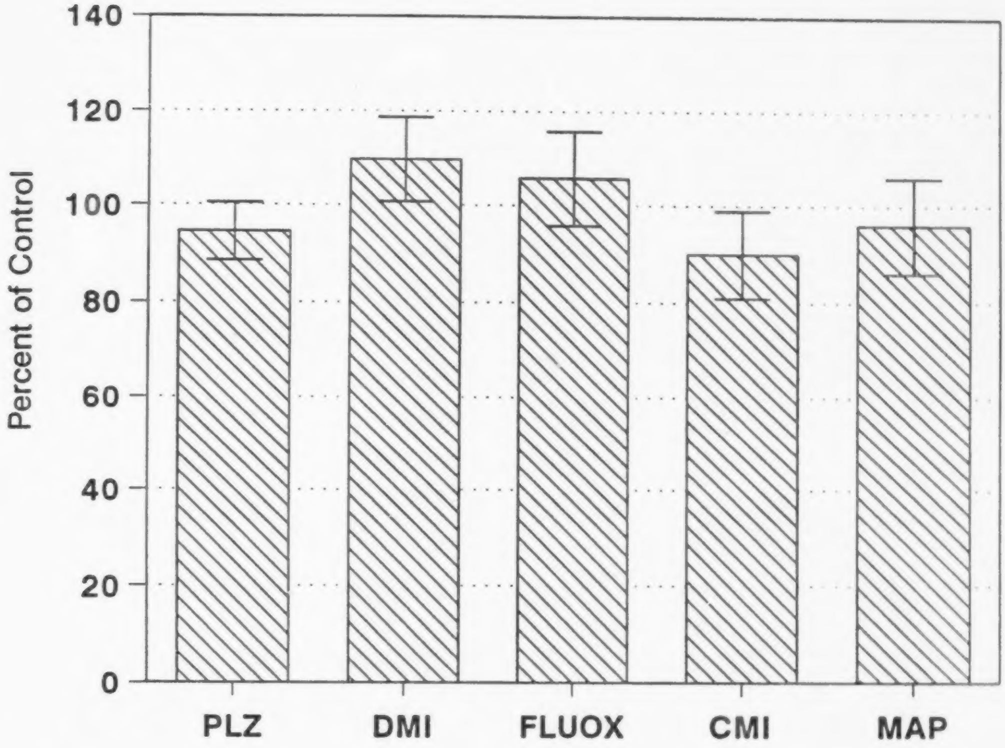


Figure 13. Results from ^{3}H-flunitrazepam binding to the benzodiazepine binding site in rat cortex. Values represent Bmax values and are expressed as percent of VEH control animals (± SEM) for PLZ, DMI, FLUOX, CMI and MAP (all 10 mg/kg/day, n=10). VEH or drugs were administered *via* osmotic minipumps for 21 days (mean control values were 97.3± 13.7 fmol/mg protein).

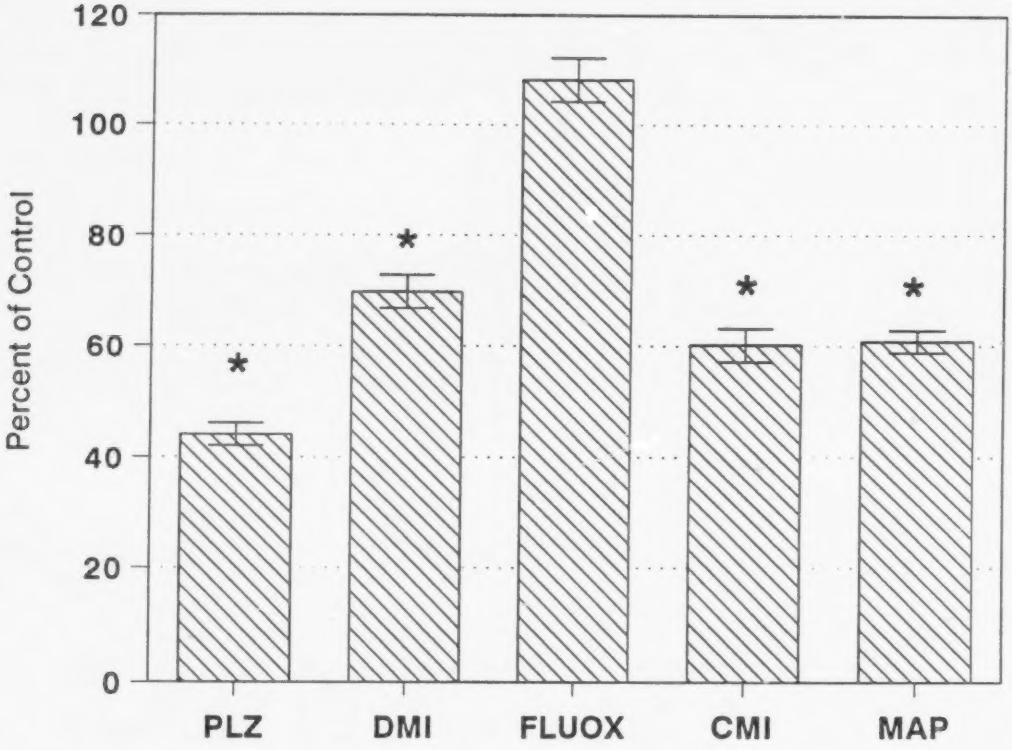


Figure 14. Results from the ^{3}H-ketanserin binding to the 5-HT$_2$ receptor in hippocampus. Values are expressed as percent of VEH control animals ± SEM for PLZ, DMI, FLUOX, CMI and MAP (all 10 mg/kg/day, n=10). VEH or drugs were administered *via* osmotic minipumps for 21 days. * denotes significant difference from VEH control (Newman-Keuls, $p<0.05$; mean control values were 1480.2± 263 dpm/mg protein).

The present investigation found a decrease in the binding to 5-HT_2 receptors, in the same animals, induced by chronic treatment with the tricyclics DMI and CMI, the tetracyclic MAP and the MAO inhibitor PLZ. No significant changes in 5-HT_2 binding were seen with chronic FLUOX treatment. These results concur with those of other investigators who also found a down-regulation of 5-HT_2 binding subsequent to chronic DMI treatment, but no change following chronic FLUOX treatment (Eison *et al.,* 1991; Lafaille *et al.,* 1991). This is an unexpected finding that is difficult to explain. One possible hypothesis for the lack of down-regulation following FLUOX treatment is that the blockade of uptake of 5-HT and the resultant increase in 5-HT in the synapse leads to an activation of terminal autoreceptors, which in turn reduce the amount of released 5-HT. The net effect would then be no reduction in post-synaptic 5-HT_2 receptor density following administration of FLUOX (Klimek *et al.,* 1994).

The present findings suggest that changes in the number or affinity of the benzodiazepine binding site associated with the $GABA_A$ receptor complex are not important effects produced by chronic administration of antidepressants. In this regard, it is interesting that Lloyd *et al.* (1989) did not observe a decrease in $GABA_A$ receptors after chronic administration of several types of antidepressants and McKenna *et al.* (1993) recently reported that chronic administration of PLZ did not result in any change in number or affinity of the $GABA_A$ receptor (labelled with ^{3}H-muscimol) associated with the benzodiazepine receptor in rat cortex. As mentioned in Chapter 1 of this thesis, recent experiments in molecular biology have shown that the $GABA_A$ receptor complex is heterogenous, existing in multiple isoforms. It is possible then, that the effects of antidepressants on the $GABA_A$-BZD receptor complex may be due to the substitution of one isoform, in a given functional $GABA_A$ receptor, with another. This substitution could change the characteristics of the drug response without necessarily changing the binding capacity. It may be neccessary then, to study levels of isoform-specific mRNAs and identify which increase and which decrease in order to see changes in $GABA_A$ receptors.

The results of the present experiments confirm the findings of several groups suggesting that down-regulation of 5-HT_2 receptors is a property shared by many antidepressants ([Baker and Greenshaw, 1989 (review)]; Eison *et al.*, 1991; Lafaille *et al.*, 1991) The area of effects of FLUOX on 5-HT receptors remains controversial, with down-regulation (Byerley *et al.*, 1986; Wamsley *et al.*, 1987), up-regulation (Dumbrille-Ross and Tang, 1983; Wamsley *et al.*, 1987; Hrdina and Vu, 1993) and no changes (Peroutka and Snyder, 1980; Fuxe *et al.*, 1983; Baron *et al.*, 1988; Eison *et al.*, 1991) having been reported.

In summary, under conditions in which it has been confirmed that the drugs of interest have been effectively delivered to the brain and in which it has been shown that, with the exception of FLUOX, they down-regulate 5-HT_2 receptors, no effects on the Bmax or K_D of ^{3}H-flunitrazepam binding were observed in the cortex of rats treated chronically with these antidepressant drugs.

The results from this study have also demonstrated that extractive acetylation under aqueous conditions is a useful procedure for analysis of CMI and separation from its N-desmethylated metabolite in brain tissue. Similar methodology has been utilized for analysis of: imipramine, desipramine and their hydroxylated metabolites in plasma and in extracts from metabolic studies with cells expressing cytochrome P450 isozymes (Coutts *et al.*, 1993; Su *et al.*, 1993); trimipramine and its N-desmethylated metabolite in urine samples (Coutts *et al.*, 1990) , and maprotiline and N-desmethylmaptrotiline in plasma samples (Drebit *et al.*, 1988). Acetylation under aqueous conditions has also been utilized as an effective procedure for extracting trace amines such as tryptamine, 2-phenylethylamine and m- and p-tyramine and amine-containing drugs such as tranylcypromine from brain tissue prior to subsequent derivatization with perfluoracylating reagents for GC-ECD or GC-MS analysis (Martin and Baker, 1977; Hampson *et al.*, 1984; Durden, 1991;Durden *et al.*, 1991; Baker *et al.*, 1993).

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CHAPTER 3

GABA-elevating effects of the antipanic/antidepressant drug phenelzine: effects of pretreatment with tranylcypromine, (-)-deprenyl and clorgyline.

(The research described in this chapter formed the basis for a submitted manuscript.)

3.1 INTRODUCTION

In recent years, there have been reports in the literature that implicate GABA systems in the etiology and pharmacotherapy of panic disorder (PD) (review: Breslow *et al.,* 1989). Phenelzine (PLZ), a non-selective MAO inhibitor is frequently prescribed for the treatment of PD (Sheehan *et al.,* 1980; Ballanger, 1986). The mechanisms by which PLZ is efficacious in alleviating the symptoms of PD have yet to be elucidated, but reports have shown that administration of PLZ to rats results not only in the expected increases of brain biogenic amines, but also in brain GABA levels (Popov and Matthies, 1969; Wong *et al.,* 1990; McKenna *et al.,* 1990; McManus *et al.,* 1992). Acute and chronic studies have indicated that this increase in brain GABA levels after PLZ treatment is due at least in part, to the inhibition of the catabolic enzyme GABA-transaminase (Popov and Matthies, 1969; McKenna *et al.,* 1991; McManus *et al.,* 1992).

Interestingly, it has been shown that by pretreating rats with tranylcypromine (TCP), another nonspecific MAO inhibitor, the GABA-elevating effects of PLZ could be blocked. The purpose of the present study was to extend this line of investigation and ascertain whether or not the blockade of PLZ's effects on GABA levels specifically requires the prior inhibition of either MAO-A or -B. To this end, the selective inhibitors of MAO-A and -B, clorgyline and (-)-deprenyl respectively have been employed and the results obtained have been compared with those obtained with TCP, a nonselective inhibitor of MAO-A and MAO-B.

3.2 MATERIALS AND METHODS

3.2.1. Animals and drug administration:

Male Sprague Dawley rats (250-300g) were randomly assigned (n=8) to either vehicle (VEH) or one of the following MAO inhibitors; TCP (1mg/kg), l-deprenyl (DEP) (1mg/kg) or clorgyline (CLG) (2mg/kg). These drug doses were based on previous investigations on the MAO-inhibiting effects of the drugs (Baker *et al.*, 1992). All injections were administered *via* the intraperitoneal (IP) cavity. One hour post injection, all animals received 10 mg/kg PLZ IP with the exception of a VEH/VEH group run as control. Four hours after PLZ injection, the rats were sacrificed by guillotine decapitation, their brains quickly removed and immediately frozen in isopentane on solid carbon dioxide. The whole brains were frozen at -80°C until analysis of brain GABA levels and of activities of MAO-A, MAO-B and GABA-transaminase (GABA-T). Additionally, another set of animals was run to ascertain the singular effects of TCP, DEP and CLG at 1 h on the same parameters as studied in the drug combination animals.

3.2.2. Analysis of MAO and GABA:

To determine the percent inhibition of the enzymes MAO-A and -B and GABA-T and the effects on brain GABA levels, the rat brains were initially halved along the midline. In counter-balanced fashion, half of the brain was homogenized in 5 volumes of 0.1N perchloric acid containing 0.05 mM ascorbic acid and 10.0 mg % EDTA. As described by Wong *et al.,* (1990) a 25 μl aliquot of the supernatant was used in a gas chromatographic assay procedure for the analysis of GABA in rat brain. To the supernatant was added 25 μl of the internal standard norleucine (250 ng). The mixture was first basified with 2.5% potassium carbonate followed by the addition of isobutychloroformate solution (5 μl/ml toluene:acetonitrile 9:1 v/v). After shaking for 10 min and centrifuging for 2 min at 2500 x g the organic phase (top layer) was aspirated and discarded. To the aqueous phase (bottom layer) was added 1.5 ml sodium phosphate buffer (2M, pH 6) and 25 μl 6 N HCL. After a brief vortex, the following were added in sequential fashion: 2.5 ml choroform, 200 μl dicyclohexylcarbodiimide (DCC) solution (5 μl/ml chloroform) and 200 μl pentafluorophenol (PfPh-OH) solution (5 μl/ml chloroform). The mixture was then vortexed for 15 min, centrifuged for 5 min and the aqueous layer (top) aspirated and discarded. The bottom chloroform layer was decanted to clean tubes and evaporated to dryness under a gentle stream nitrogen gas. The residue was reconstituted in 300 μl of toluene and washed briefly with 500 μl of distilled water; after a rapid centrifugation, the organic (top) layer removed for

GC analysis. Samples were injected *via* an automatic sampler and chromatographic separation was accomplished using the following automatic temperature program: an initial temperature of 100°C was maintained for 0.5 min; this was increased to 200°C at a rate of 25°C/min. After maintaining at 200°C for 0.5 min, the temperature was then increased to 230°C at a rate of 3°C/min. The chromatographic column used was a fused silica capillary column (25m x 0.32mm x 1.05 μm film thickness), cross-linked 5% phenylmethylsilicone phase.

A calibration curve was prepared with each assay run to permit analysis of the quantity of the neurochemical of interest. This curve was constructed by adding known, varying amounts of authentic standards and a fixed amount (the same added to the tissue supernatants) of internal standard to a series of tubes containing supernatants from homogenates of drug-naive brains and carrying these tubes through the assay procedure in parallel with the sample tubes.

The other brain half was homogenized in ice-cold distilled water, and aliquots removed for analysis of MAO-A and -B activity employing a modified version of the radiochemical method of Wurtman and Axelrod (1963) (described in Chapter 2) and GABA-T activity. For the GABA-T assay, a 1 ml aliquot of the homogenate was mixed with 20 volumes of a modification of the medium described by Palfreyman *et al.* (1978). Composition of the medium was: glycerol (20% v/v), Triton X-100 (0.13% v/v), reduced glutathione (100 μM), pyridoxal 5-phosphate (1 μM), Na_2EDTA (1mM), dipotassium monophosphate

(5 mM) and sufficient glacial acetic acid to bring the pH value to 7.2-7.4. A 10 μl aliquot of this mixture was then utilized to assay for GABA-T activity using the radiochemical procedure described by Sterri and Fonnum (1978). This procedure involves the preparation of an incubation medium containing 1 μl ^{3}H-GABA (specific activity of 40 Ci/mmol), 7.5 μl 100 mM GABA, 15 μl 50 mM α-ketoglutarate, 15 μl 10 mM nicotinamide adenosine dinucleotide, 15 μl 10 mM 2-aminoethylisothiouronium bromide, 1 μM pyridoxal phosphate, 60 μl distilled water and 37.5 μl 50 mM Tris buffer (pH 7.9). To 1.5 ml microfuge tubes placed on ice, 10 μl of tissue homogenate (10 μl of distilled water to blanks) and 20 μl of the incubation medium were added. The tubes were incubated at 37°C for 30 min at which time 100 μl of tri-n-octylamine were added. The mixture was vortexed briefly and centrifuged at 1000 x g for 2 min. A 35 μl aliquot of the top layer was removed and added to a scintillation vial containing 4 ml of scintillation fluid. Radioactivity was measured in a liquid scintillation counter after allowing the samples to sit for 12 hours.

3.2.3. Statistical Analysis:

Data were analyzed by two-way analysis of variance followed by the Newman-Keuls multiple comparison test on significant main effects and interactions. A two-tailed probability distribution was used, and the general convention of a probability value of $p<0.05$ was used to establish statistical significance.

3.3 RESULTS

Displayed in Figure 15 are the results of the brain GABA analysis from animals receiving the drug combination and in Figure 16 the results from the single drug treatments. Animals treated with an initial injection of VEH, DEP or CLG followed by PLZ showed significantly increased GABA levels as compared to the VEH/VEH control rats (interaction $F(1,31)=7.63$). The TCP/PLZ group had brain GABA levels similar to those of the VEH/VEH group, i.e. the GABA-elevating effects of PLZ were completely blocked. Pretreatment with DEP resulted in a significant reduction of the GABA-elevating effect of PLZ, while pretreatment with CLG did not.

The data from the one hour control animals shows that neither TCP, DEP nor CLG had significant effects on GABA levels when administered alone.

Figure 17 is a graphical representation of the data from the GABA-T analysis from animals receiving the drug combination and Figure 18 is the results from single drug treatments. The data show that PLZ in combination with VEH, DEP and CLG significantly inhibited GABA-T activity compared to the VEH/VEH controls (interaction $F(1,31)= 6.87$). The animals treated with TCP prior to PLZ were no different than the VEH/VEH controls with respect to GABA-T activity ($p>0.05$), ie TCP had competely reversed the inibition of GABA-T produced by PLZ.

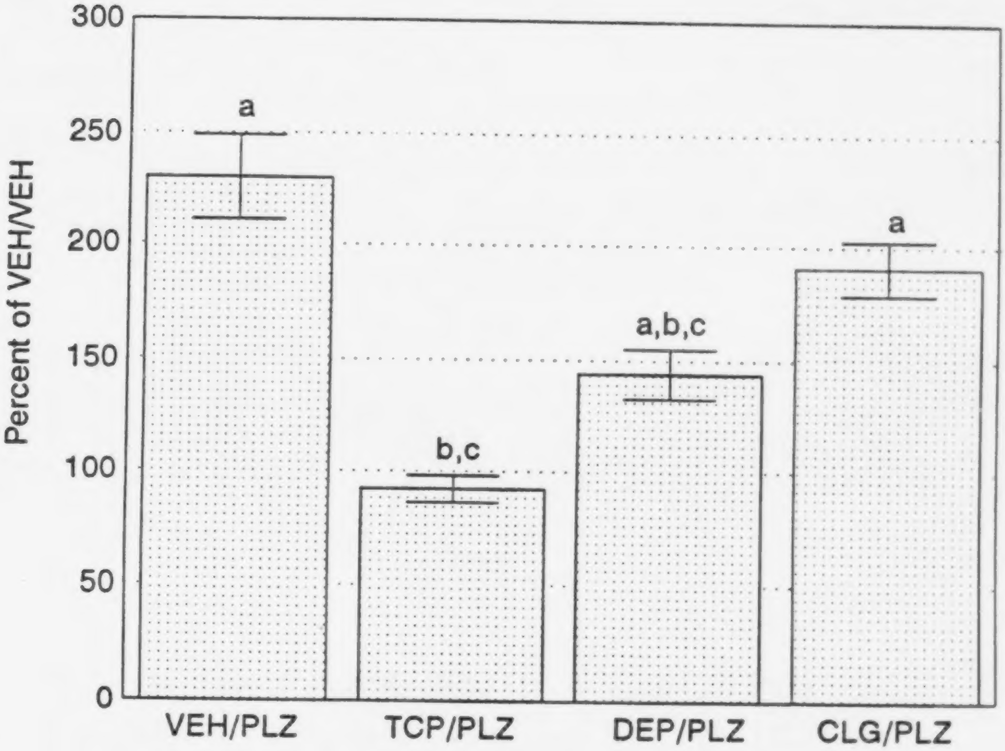

Figure 15. Mean levels of GABA (± SEM) in rat brain following drug combination treatment. Histograms represent percent of VEH/VEH control values. a, significantly different from VEH/VEH; b, significantly different from VEH/PLZ; c, significantly different from CLG/PLZ. Drug doses were PLZ-10 mg/kg; TCP-1 mg/kg; DEP-1mg/kg; CLG-2 mg/kg (all IP, n=8). Mean control GABA levels were 254.8± 20.6 μg/g tissue).

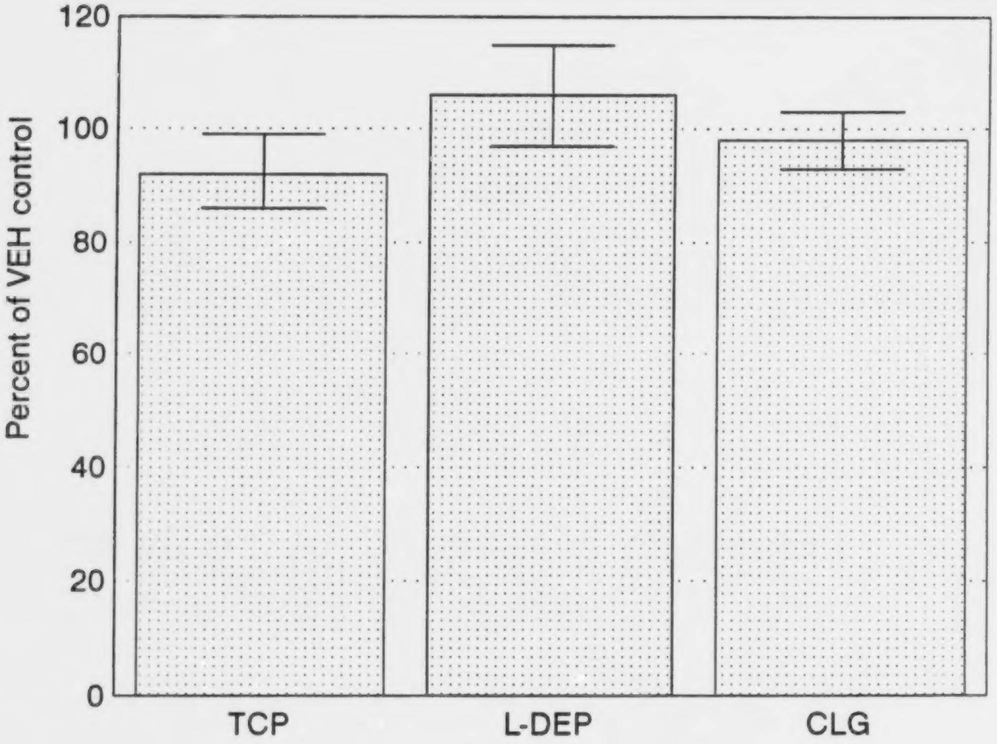

Figure 16. Mean levels of GABA (± SEM) in rat brain following 1 h treatment with TCP, DEP or CLG. Drug doses are the same as those in Figure 15. Histograms represent percent of VEH (mean control GABA levels were 232.2± 19.2 μg/g tissue).

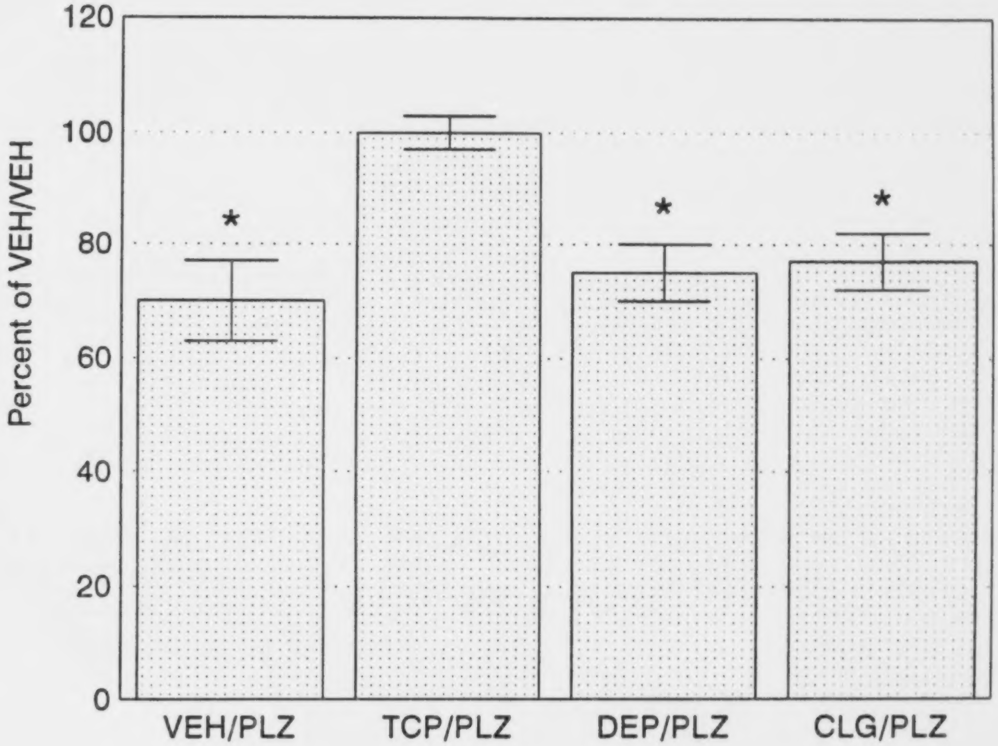

Figure 17. Mean GABA-T activity (± SEM) in rat brain following drug combination treatment. Drug doses are the same as those shown in Figure 15. Histograms represent percent of VEH/VEH. * Denotes significantly different from VEH/VEH.

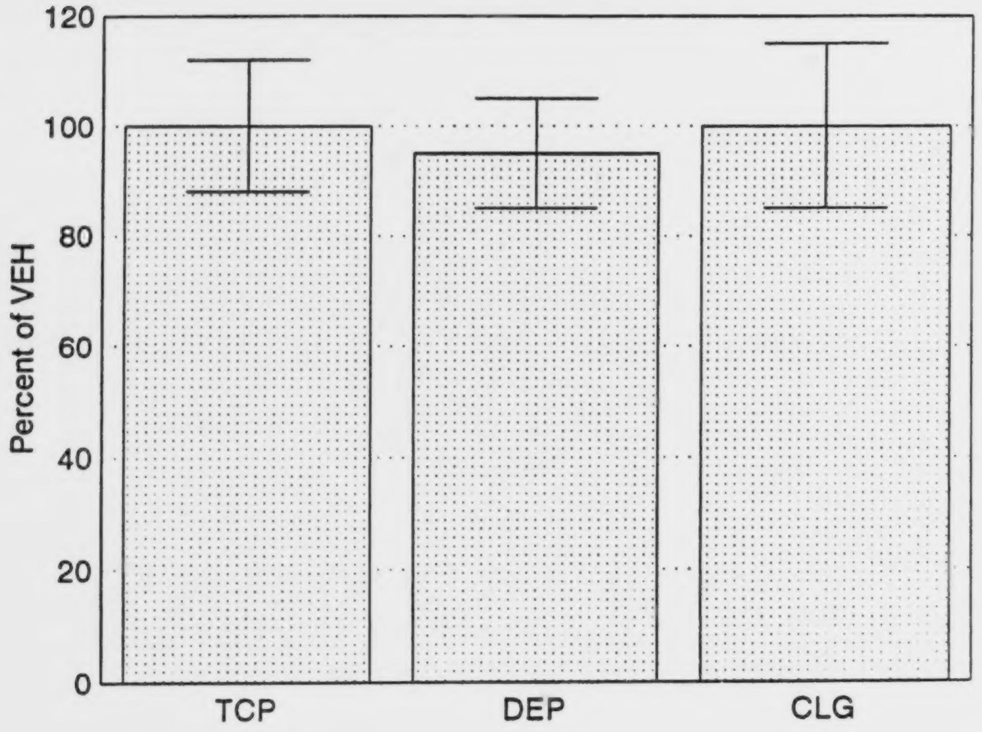

Figure 18. Mean GABA-T activity (± SEM) in rat brain following 1 h treatment with TCP, DEP or CLG. Drug doses are the same as those reported in Figure 15. Histograms represent percent of VEH.

The data analysis from the one hour control animals receiving single injections of DEP, TCP and CLG revealed no significant differences in the activity of GABA-T ($p>0.05$).

As shown in Figure 19, at the doses employed, TCP significantly decreased activity of both MAO-A and-B while, as expected, CLG significantly decreased only MAO-A and DEP significantly decreased only MAO-B activity ($p<0.05$).

3.4 DISCUSSION

This investigation showed that the VEH/PLZ treatment more than doubled brain GABA levels as compared to the VEH/VEH controls. This increase was effectively blocked by pretreatment with the nonselectve MAO inhibitor TCP. DEP, the selective inhibitor of MAO-B, also decreased the GABA-elevating effect of PLZ, but to a lesser extent than TCP even though inhibition of MAO-B was similar with DEP and TCP. Only prior treatment with TCP was effective in completely blocking the GABA-elevating effects of PLZ. However, pretreatment with DEP did significantly reduce the GABA-elevating effects of PLZ (compare Figure 15, VEH/PLZ and DEP/PLZ). With the exception of PLZ (as seen in the VEH/PLZ group) no drug group produced a significant inhibition of GABA-T activity when administered in isolation.

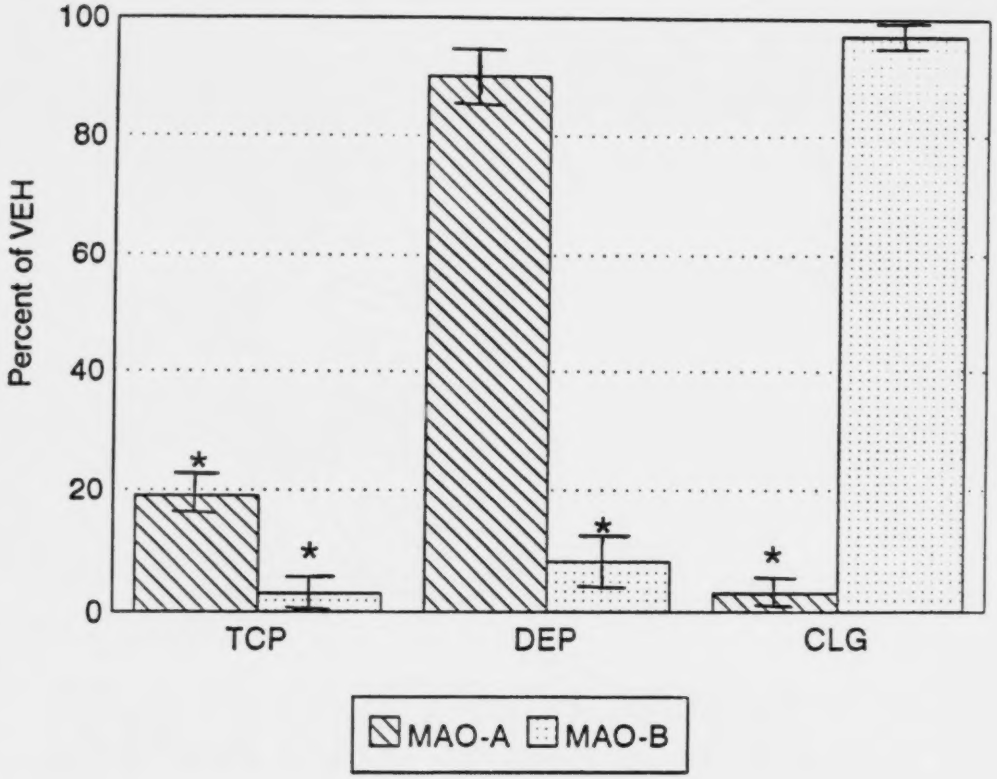

Figure 19. Mean MAO-A and -B activity (± SEM) in rat brain following 1 h treatment with TCP, DEP or CLG. Drug doses are the same as those reported in Figure 15. Histograms represent percent of VEH. * Denotes significantly different from VEH. The degree of inhibition of MAO-B by TCP and DEP was not significantly different between the two drugs.

The mechanism for the interaction between PLZ and GABA is not yet known. PLZ is an unusual drug in that it acts not only as an inhibitor of, but is also as a substrate for MAO (Clineschmidt and Horita, 1968; Tipton and Spire, 1972). Popov and Matthies (1969), based on their observation that pretreatment of rats with a high dose of the MAO inhibitor TCP blocked the effects of PLZ on brain GABA, suggested many years ago that a metabolite of PLZ may be responsible for the GABA-elevating effect of PLZ, and the present results support their suggestion. At present, the structure(s) of that/those metabolite(s) responsible is unknown, but the present findings that DEP blocked the elevating effects of PLZ with greater efficiency than did clorgyline, suggest that MAO-B may play a more important role in its (their) formation than MAO-A. The amine β-phenylethylamine (PEA) may be a candidate for the relevant metabolite of PLZ since it is a known metabolite of PLZ (Baker *et al.*, 1989; Dyck *et al.*, 1985) and is an excellent substrate for MAO-B. Experiments on the interactions of PEA with GABA in brain tissue *in vitro* and *in vivo* are now being set up by other researchers in the Neurochemical Research Unit.

The results presented here also suggest that the elevation of GABA produced by PLZ may involve more than just a simple inhibitor of GABA-T. (-)-Deprenyl was able to partially reverse the GABA-elevating effect of PLZ, but did not cause a significant reversal of the GABA-T inhibiting effect of PLZ. It is also of interest, that PLZ more than doubled GABA levels in brain while inhibiting GABA-T by only 30%, a finding which is in agreement with those of Popov and

Matthies (1969) and Baker *et al.* (1991). In support of the notion that PLZ's effects involve more than just the inhibition of GABA-T is the recent finding that glutamine levels in the hypothalamus are found to be markedly decreased following acute PLZ administration (Sloley, Baker and Paslawski; personal communication). Glutamine is formed after glial uptake of GABA from the synaptic cleft. The enzyme involved in glutamine formation is glutamine synthetase. The reduction of glutamine following PLZ treatment suggests that in addition to inhibiting GABA-T activity, PLZ also inhibits glutamine synthetase, and this action may contribute to the dramatically elevated GABA levels. It is also possible that the transport mechanism for GABA uptake into neural and/or glial cells is in some manner blocked by PLZ. Further research on the effects of PLZ on glutamine synthetase and GABA uptake are now underway by other researchers in the Neurochemical Research Unit.

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CHAPTER 4

EFFECTS OF TWO SUBSTITUTED HYDRAZINE MAO INHIBITORS ON NEUROTRANSMITTER AMINES, GABA, AND ALANINE IN RAT BRAIN

[The work described in this chapter formed the basis for a published paper (J Pharm Sci, 82:934-937)]

4.1 INTRODUCTION

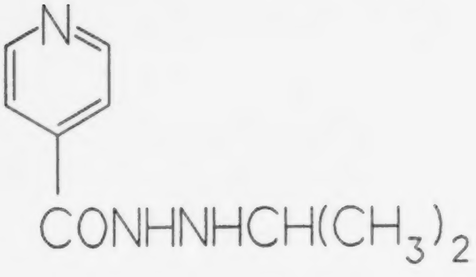
The area of mechanisms of action of antidepressant and antipanic drugs remains controversial. Accumulated evidence suggests a role for brain GABA in the etiology and pharmacotherapy of depression and panic disorder (Lloyd *et al.,* 1989; Breslow *et al.,* 1989). Phenelzine (PLZ), one of the most commonly used MAO-inhibiting antidepressants, is also used to treat panic disorder (Sheehan *et al.,* 1980; Ballenger, 1986), and has been reported to elevate brain concentrations of the amino acids GABA (Popov and Matthies, 1969; Perry and Hansen, 1973; Baker *et al.,* 1991) and alanine (ALA) (Wong *et al.,* 1990). Previously, it was shown in our laboratory that N^2-acetyl-PLZ, formed by derivatizing the free hydrazine group of PLZ, retained MAO-inhibiting properties but did not elevate brain GABA or ALA levels at doses at which there was marked inhibition of MAO (McKenna *et al.,* 1991). Currently, there is little known regarding the role of ALA in the central nervous system or its possible involvement in the etiology of depression or panic disorder. It has been reported that ALA is present in various species at concentrations 25-60% of those of GABA, and that administration of the convulsant pentamethylenetetrazole results in increased brain ALA levels (Clarke *et al.,* 1989). Further, ALA is metabolically related, *via* transamination, to pyruvate and lactate. Lactate has been shown to produce panic attacks in some individuals (Shear, 1986). It is possible that the increase in brain ALA levels may reflect a decrease in lactate formation, and that this may contribute to the antipanic effects of PLZ.

The purpose of the next set of investigations described in this thesis was to extend the findings on the effects of PLZ on GABA and ALA to two other substituted hydrazine MAO-inhibiting antidepressants, iproniazid (IPR) and nialamide (NIAL) (Figure 20). That is, it was important to ascertain whether the GABA elevation seen with the nonspecific MAO inhibitor PLZ was specific to PLZ. To this end, acute dose-response and time-course studies of both drugs were undertaken to examine their effects on MAO-A and -B activity; on brain levels of NA, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT, 5-hydroxyindole-3-acetic acid (5-HIAA), GABA and ALA; and on the activity of the enzymes GABA-transaminase (GABA-T) and ALA-transaminase (ALA-T).

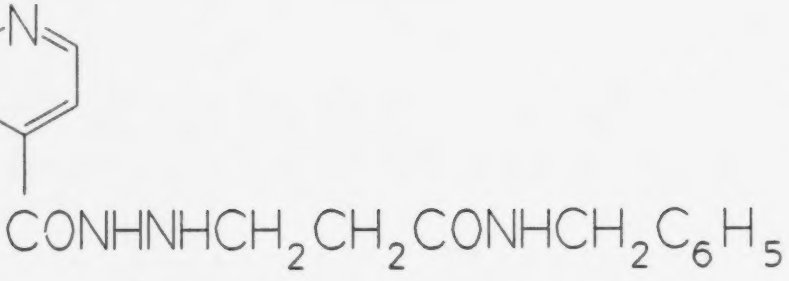
4.2 MATERIALS AND METHODS

4.2.1. Animals and Drug Administration:

For the time study, male Sprague Dawley rats (250-300 g) with free access to food and water were randomly assigned to groups (n=6) receiving IPR (50 mg/kg IP), NIAL (100 mg/kg IP) or physiological saline vehicle. Animals were sacrificed by decapitation 1, 2, 4, 8, 16 or 24 h after injection. The brains


N
CONHNHCH(CH3)2

iproniazid


N
CONHNHCH2CH2CONHCH2C6H5

nialamide

Figure 20. Chemical structures of IPR and NIAL.

were removed, frozen on solid carbon dioxide and subsequently stored at -80°C until time of analysis. The dose-response analysis consisted of rats randomly assigned to IPR, NIAL (25, 50 or 100 mg/kg IP for each drug) or vehicle groups and sacrificed 4 h later. This time interval was chosen since it was one at which there was extensive inhibition of MAO and since it is the time interval at which PLZ was shown in previous studies to cause maximum elevation of brain GABA and ALA levels. Previous dose-response studies with PLZ had also been conducted at this time interval (Baker *et al.*, 1991; Wong *et al.*, 1990).

4.2.2. Analysis:

Frozen brains were weighed and homogenized in 5 volumes of ice-cold distilled water and portions removed for analyses of MAO, GABA-T and ALA-T. To the remainder of the homogenate was added 1/10th the volume of 1.0 N perchloric acid containing 100 mg % EDTA and 0.5 mM ascorbic acid. The homogenate was then centrifuged at 12,000 x g for 15 min at 4°C to remove the protein precipitate. Aliquots of the supernatant were used for analysis of concentrations of GABA, ALA and the biogenic amines and their acid metabolites.

4.2.3. Neurotransmitter amines and acid metabolites:

Levels of NA, DA, DOPAC, HVA, 5-HT and 5-HIAA were determined using high pressure liquid chromatography with electrochemical detection. The methodology and apparatus employed were as described in Baker *et al.* (1987). Specifically, to 150 μl aliquots of the clear supernatant obtained were added 200 ng of internal standard. Aliquots (15 μl) of this mixture were then each injected into the HPLC system described earlier. Calibration curves of known concentrations of the amines and acid metabolites were run in parallel to each group of samples. Linearity was determined and correlation coefficients of >0.99 were obtained routinely.

4.2.4. Amino acids:

GABA and ALA levels were determined using gas chromatography with an electron-capture detector. This method, described by Wong *et al.* (1990) (details given in Chapter 3), involves the conversion of the amino acids into N-isobutyloxycarbonyl, pentafluorophenyl ester derivatives prior to gas chromatographic analysis.

4.2.5. Enzymes:

Brain MAO activity was analyzed employing the modified version of the radiochemical procedure of Wurtman and Axelrod (1963). This assay uses ^{14}C-5-HT and ^{14}C-β-phenylethylamine as substrates for MAO-A and -B, respectively (detailed in Chapter 2). The assay procedure used for measurement of activity of GABA-T and ALA-T was that of Sterri and Fonnum

(1978), which relies on the selective extraction of labelled acids formed from GABA or ALA using a liquid anion exchanger (as described in Chapter 3).

4.2.6. Statistical analysis.

Statistical analysis consisted of two-way analysis of variance followed by Newman-Keuls tests for multiple pairwise comparisons. A significance level of $p<0.05$ was chosen to identify significant differences.

4.3. RESULTS

Analysis of MAO activity revealed a strong inhibition of MAO-A and -B by IPR at all doses and times. Treatment with NIAL resulted in a dose-related graded inhibition of MAO-A and -B, with a slightly stronger inhibition of MAO-A (70-89%) than MAO-B (38-69%) at all doses and times (Figures 21 and 22).

Figure 23 shows that all doses of IPR and NIAL resulted in significantly increased levels of NA, DA and 5-HT and decreased levels of the metabolites DOPAC, HVA and 5-HIAA ($p<0.05$). Time-course analysis revealed that both drugs initially produced graded increases of NA, DA and 5-HT followed by a graded decrease to 24 h for DA and 5-HT. The metabolites DOPAC, HVA and 5-HIAA were significantly decreased over time as compared to controls ($p<0.05$) (Figure 24). The highest levels of amines for both drugs were

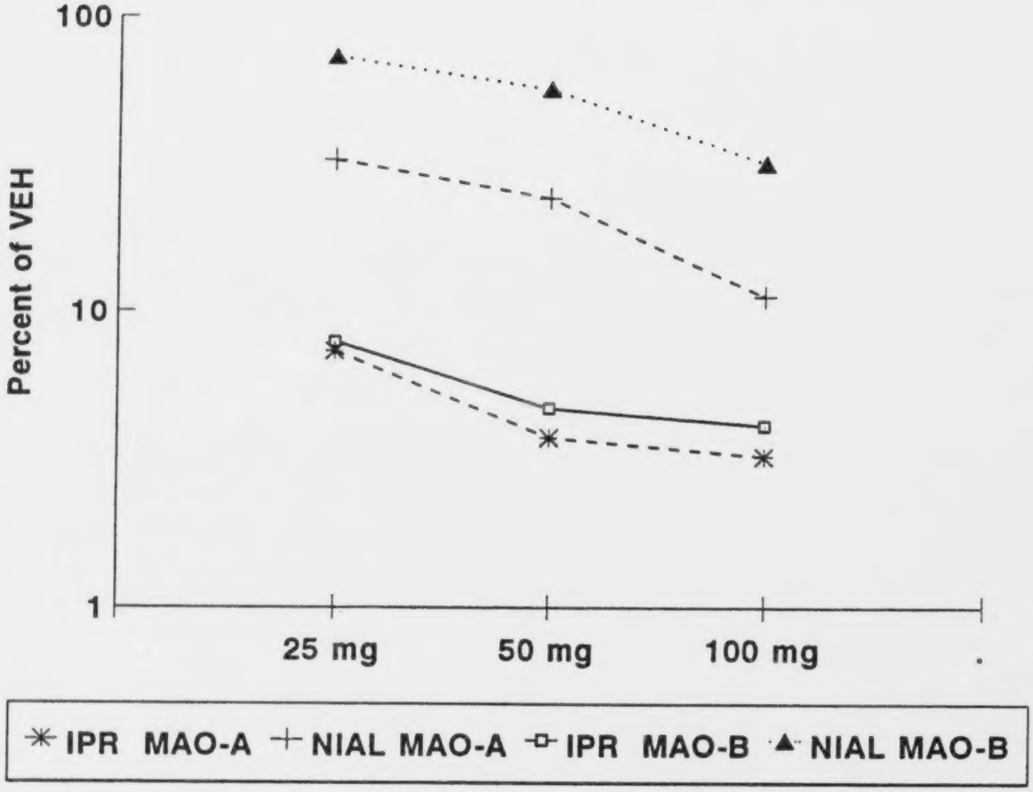


Figure 21. Log-linear plot of MAO-A and -B activity in rat whole brain 4 h after IP injection of IPR (25, 50 or 100 mg/kg). Values are expressed as mean percent of VEH. SEMs for all groups were less than 10% of the group mean.

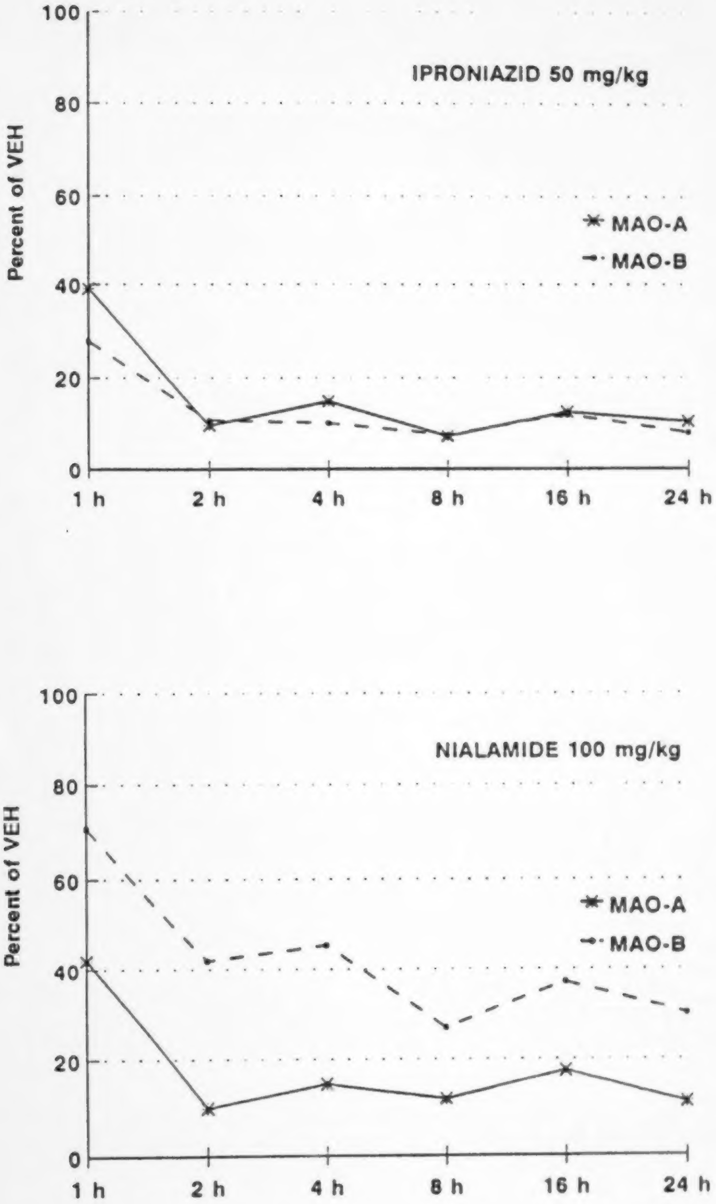


Figure 22. MAO-A and -B activity in rat whole brain 1, 2, 4, 8, 16 and 24 h after IP injection of IPR (50 mg/kg) or NIAL (100 mg/kg). Values are expressed as mean percent of VEH at the same time intervals. SEMs for all groups were less than 10% of the group mean.

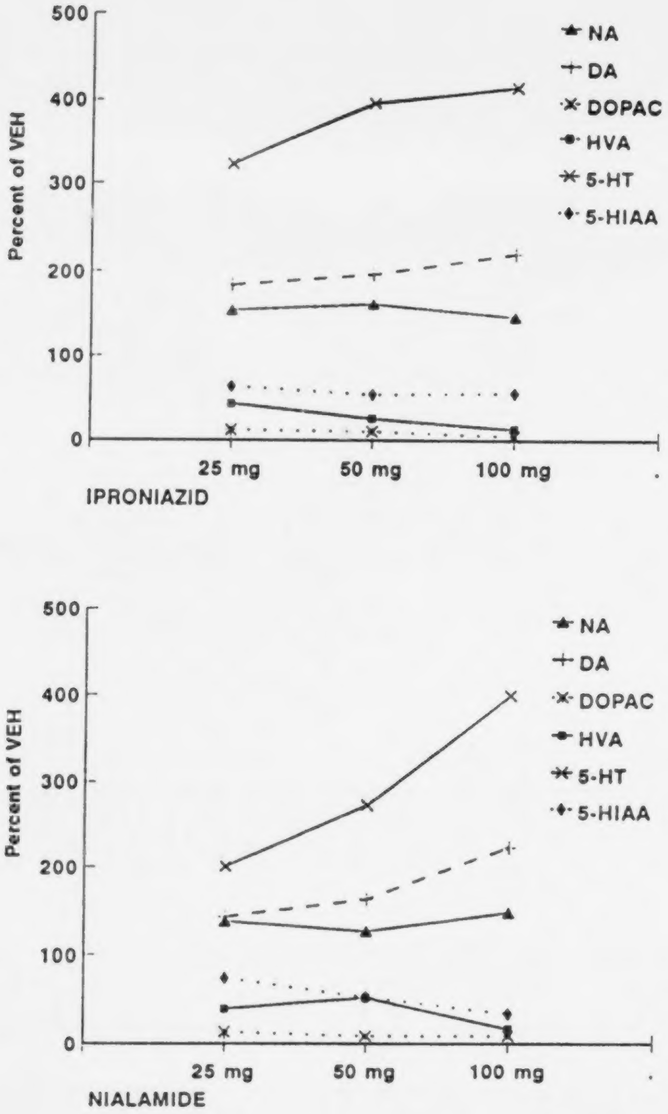


Figure 23. Whole brain levels of neurotransmitter amines and acid metabolites 4 h after IP injection of IPR (25, 50 or 100 mg/kg) and NIAL (25, 50 or 100 mg/kg). Values are expressed as mean percent of VEH. Control levels (ng/g) were NA (293.2± 15.5), DA (743.5± 23.4), DOPAC (196.7± 11.9), HVA (80.3± 1.6), 5-HT (428.3± 37.7), 5-HIAA (448.5± 20.9) [n=12]. SEMs all less than 10% of the group mean.

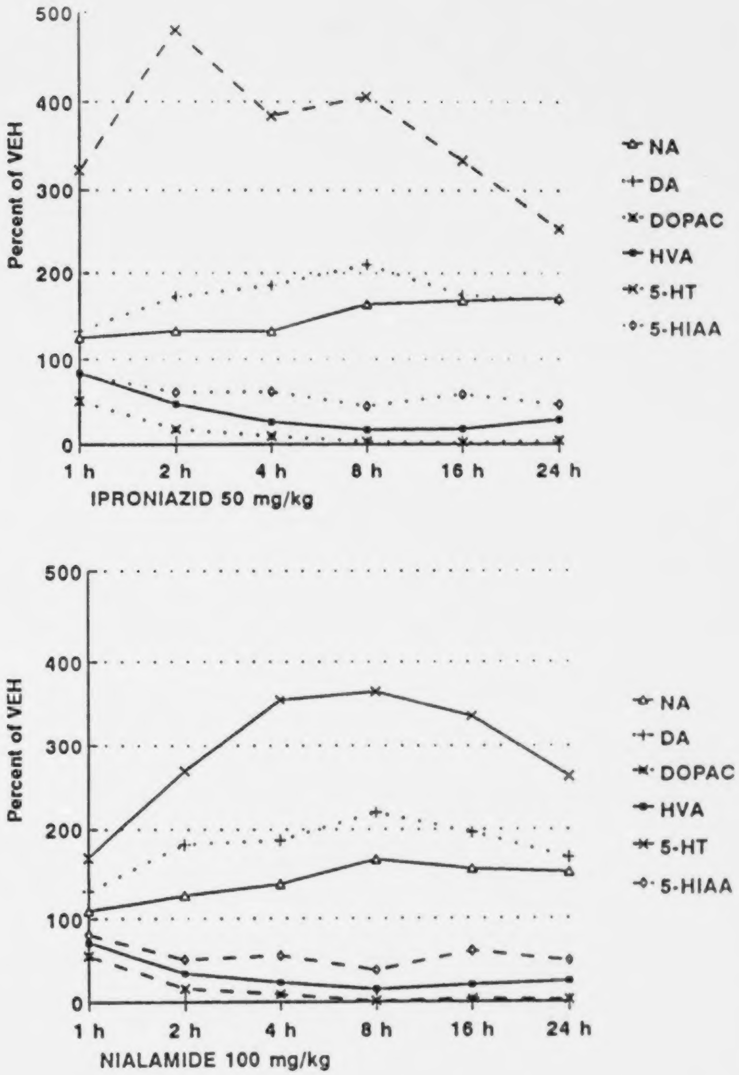


Figure 24. Whole brain levels of neurotransmitter amines 1, 2, 4, 8, 16 and 24 h after IP injection of IPR (50 mg/kg) or NIAL (100 mg/kg). Values are expressed as mean percent of VEH at the same time interval. These VEH values were not significantly different from the values reported in Figure 23. SEMs for all groups were less than 20% of the group mean.

typically found at 8 h for NA and DA and 2-4 h post injection for 5-HT. The lowest levels for the acid metabolites were typically found at 8 h for both drugs.

No significant changes from control values were evident in whole brain GABA or ALA levels for any of the doses or times tested with IPR or NIAL. Similarly, neither drug resulted in significant changes of the metabolic enzymes GABA-T or ALA-T at any dose or time ($p>0.05$ compared to controls). These results are presented in Figures 25 and 26 respectively.

4.4 DISCUSSION

The present study suggests that a free hydrazine group is critical for elevation of brain GABA and ALA levels but not for inhibition of MAO activity. The results of the current study are consistent with the previous findings on N^2-acetyl-PLZ (McKenna *et al.,* 1991). Specifically, when the free hydrazine group is further substituted as in the case of N^2-acetyl-PLZ, IPR and NIAL, the compounds retain their ability to inhibit MAO, but do not elevate GABA and ALA levels even at doses at which MAO is markedly inhibited. The elevation of GABA and ALA levels following treatment with PLZ is thought to be due, at least in part, to an inhibition of the catabolic enzymes GABA-T and ALA-T (Popov and Matthies, 1969; Baker and Martin, 1989; McManus *et al.,* 1992). At the doses tested in the present study, the substituted hydrazine compounds were

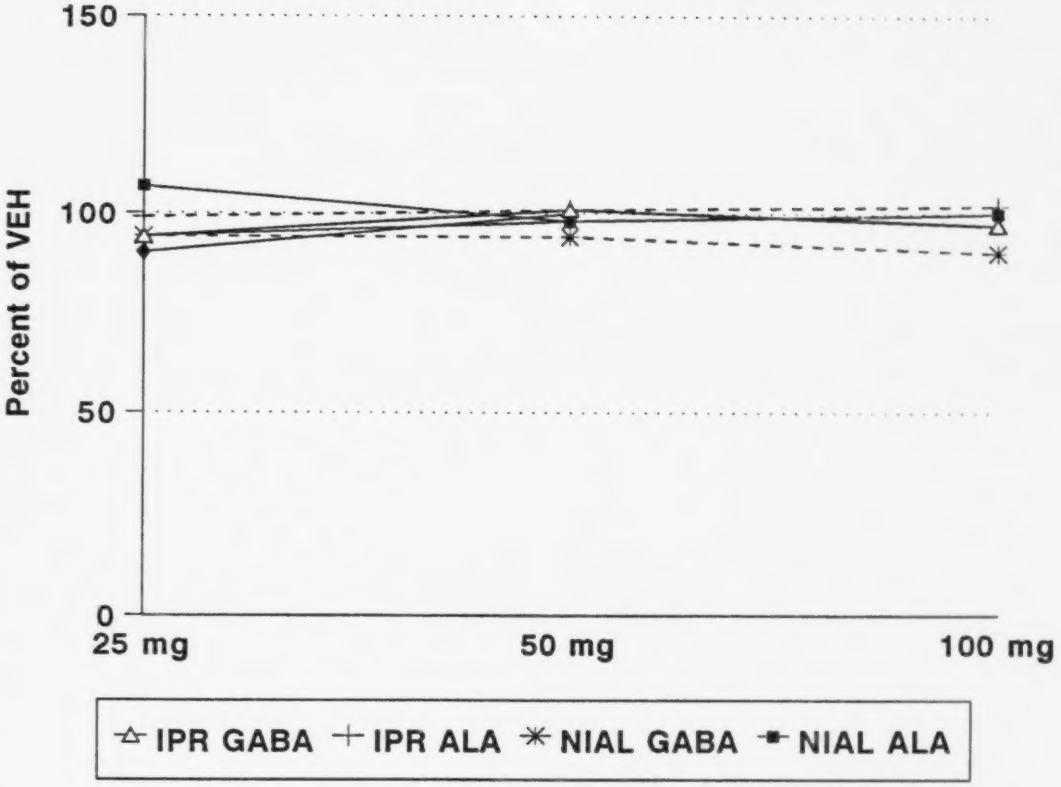


Figure 25. Whole brain levels of GABA and ALA 4 h after injection of three doses each of IPR or NIAL. SEMs for all groups were less than 10% of the group mean. VEH values were 253.0± 19.7 μg/g tissue and 51.8± 3.6 μg/g tissue for GABA and ALA respectively.

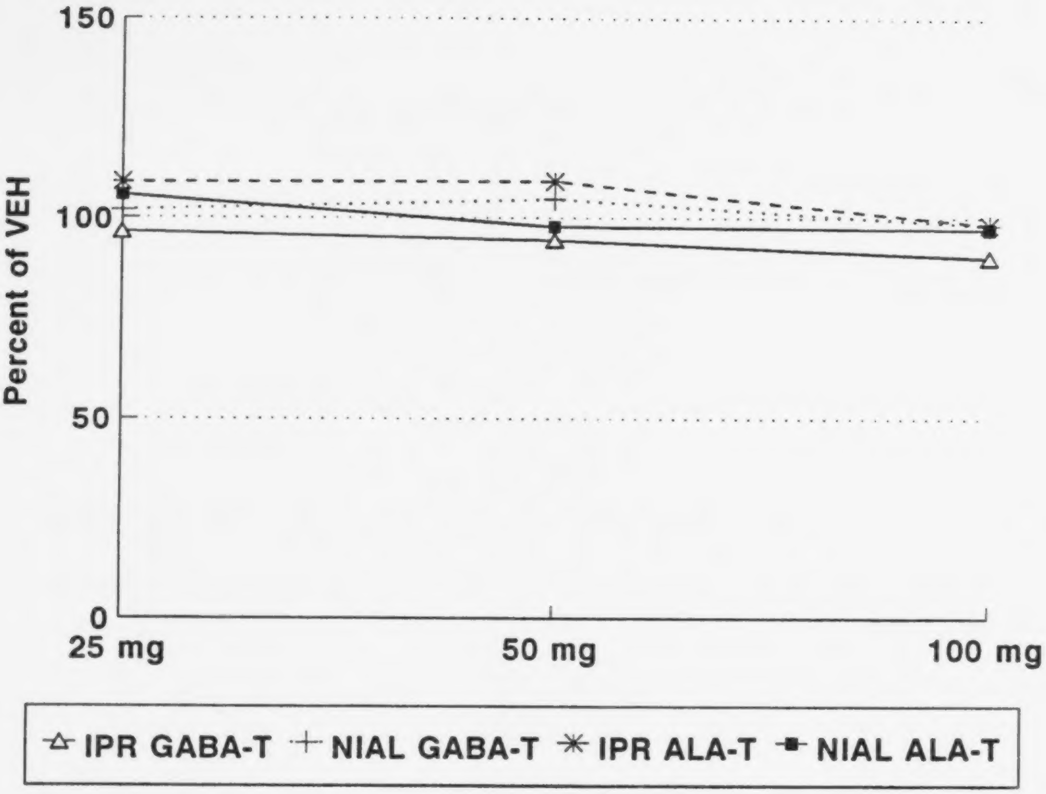


Figure 26. Whole brain levels of GABA-T and ALA-T enzymatic activity 4 h after IP injection of three doses each of IPR or NIAL. SEMs for all groups were less than 10% of the group mean.

found to have no effect on these two enzymes. These findings are also consistent with the effects of N^2-acetyl-PLZ where activities of GABA-T and ALA-T were similarly unaffected (McKenna *et al.,* 1992).

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CHAPTER 5

Comparison of the effects of phenelzine and vigabatrin on GABA, alanine, GABA-transaminase and alanine-transaminase in rat brain.

(The work reported in this chapter forms the basis of a manuscript which is in preparation).

5.1 INTRODUCTION

The GABAergic system plays an important role in brain function and, indeed, GABA is the most widely distributed inhibitory chemical transmitter in the central nervous system (Fonnum and Storm-Mathison, 1978). The rapid removal of the amino acid from the immediate area of the relevant receptor terminates GABA transmission. This is achieved through the action of a sodium-dependent GABA pump located in the presynaptic membrane and in surrounding glial cell outer membranes (Martin, 1976). The catabolism of GABA is due to the combined catalytic action of two enzymes - GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). These enzymes convert GABA to succinate *via* the intermediate succinic semialdehyde. During the reaction, α-ketoglutarate acts as an amino group acceptor and, as a result, is converted to glutamate. Pyridoxal 5-phosphate is required as a cofactor for the initial transamination step, and NAD+ is a coenzyme for the accompanying oxidation (Tunnicliff, 1989).

Many compounds are now known that can inhibit the catalytic activity of GABA-T. The majority of these substances can be classified as structural analogues of GABA and behave as competitive inhibitors of the enzyme. Various inhibitors act *via* interference with GABA binding to the enzyme. Examples are hydroxylamine (Baxter and Roberts, 1961) and aminooxyacetic acid (Wallach, 1960), which is known to cause seizures due to the concurrent inhibition of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) (Wood, 1975). Other inhibitors act by interfering with cofactor binding; an example is aminooxyacetic acid-pyridoxal 5-phosphate which binds with high affinity to the enzyme (Churchich, 1982). Still other compounds act to inhibit GABA-T by forming a covalent bond with an essential amino acid residue, thus inactivating the enzyme; these inhibitors are termed "suicide inhibitors" (Tunncliff, 1989)

An example of such a suicide inhibitor is γ-vinylGABA or vigabatrin (VIG). VIG is an enzyme-activated irreversible inhibitor of GABA-T that has anticonvulsant activity in both animals (Meldrum and Horton, 1978) and epileptic patients (Rimmer and Richens, 1984; Kalviainen *et al.,* 1993). Recent reports have shown that VIG administered to rats 18 h prior to death produced an almost 3-fold increase in GABA levels of rat cortex and spinal cord (Neal and Shah, 1990). A similar elevation of brain GABA was found in rats chronically (17 days) treated with VIG (Neal & Shah, 1990). This drug was also reported to significantly increase brain GABA levels in mice maximally at 4 h after IP injection (Schechter *et al.,* 1979). This time course of maximal GABA elevation is similar to that seen with PLZ, an MAO inhibitor that elevates GABA apparently by inhibiting GABA-T (Popov and Matthies, 1969; McKenna *et al.,* 1991; McKenna *et al.,* 1992). In addition, PLZ is classified as a suicide inhibitor of MAO, binding to that enzyme *via* a covalent bond. MAO is a flavoprotein with the cofactor flavin adenine dinucleotide (FAD) bound to a cysteine residue (Nagy & Salach, 1981). Upon combination with an amine substrate, MAO is reduced. The initial step in the interaction between an inhibiting drug and the enzyme involves the formation of a reversible complex, subject to competitive inhibition. The second step involves the generation of a reactive species, which then forms a covalent bond with a group on the enzyme, leading to irreversible inhibition (McDaniel, 1986).

The elevation of brain GABA levels may have clinical relevance in the treatment of panic disorder (PD). This illness can be chronic or acute, and is often complicated by depression, phobias and obsessions. It has been reported to affect at least 10% of the population (Keller and Hanks, 1993). Recently, it has been suggested that GABA is involved in the action of many antipanic drugs (Breslow *et al.,* 1989), and PLZ , a GABA-elevating MAO inhibitor, is currently one of the drugs commonly used to treat panic disorder (Sheehan *et al.,* 1980; Ballenger, 1986).

Given that both PLZ and VIG elevate GABA levels, with maximal elevation occurring at 4 h post administration, it was of interest to conduct an investigation comparing the *ex vivo* effects of VIG and PLZ on a number of parameters. These parameters included the drugs' effects on brain MAO-A and -B, GABA-T and GABA levels. Concurrently, levels of brain alanine (ALA) and the catabolic enzyme ALA-transaminase (ALA-T) were also measured, as previous reports in the literature have shown that PLZ also increases brain ALA levels (Wong *et al.,* 1990b, McKenna *et al.,* 1991). Additionally, the effects of preadministration of TCP on these same parameters were investigated. This latter study was of interest as it has been reported that TCP blocks the GABA-elevating effect of PLZ (Popov and Matthies, 1969; McKenna and Baker, 1992; Chapter 3 of this thesis).

5.2. MATERIALS AND METHODS

5.2.1. Part 1. Single Drug Treatment

5.2.2. Drug Administration:

Male Sprague Dawley rats weighing 250-300 g with free access to food and water were randomly assigned (n=6) to one of the following groups: VIG (1000mg/kg); PLZ (10mg/kg) or distilled water vehicle (VEH). These drug doses were chosen based on preliminary experiments in the Neurochemical Research Unit which showed that a 1000 mg/kg dose of VIG was required to give the same GABA elevation as 10 mg/kg of PLZ. Animals were given an initial IP injection of the appropriate drug in counterbalanced fashion and were sacrificed by decapitation 4 h post injection. The brains were then rapidly removed and immediately frozen in ice-cold isopentane on solid carbon dioxide. Subsequently, the brains were stored at -80°C until time of analysis.

5.2.3. *Analysis:*

At the time of analysis, the frozen brains were halved along the midline , and one half removed for analysis of MAO, GABA-T and ALA-T, while the other half was homogenized in 0.1 N perchloric acid containing 100 mg % EDTA and 0.5 mM ascorbic acid. This homogenate was then centrifuged at 12,000 x g for 15 min at 4°C to remove the protein precipitate. Aliquots of the supernatant were then used for analysis of GABA and ALA levels employing the methodology of Wong *et al.* (1990a) as described previously. The other half brain was homogenized in 5 volumes of distilled water and aliquoted for analysis of MAO-A and -B and GABA-T and ALA-T using the methodologies of Wurtman and Axelrod (1963) and Sterri and Fonnum (1978) described previously.

Statistical analysis consisted of applying one-way and two-way ANOVA (for the drug combinations) to the treatment means of all groups. *Post hoc* Newman-Keuls comparisons were applied to the data showing significant treatment main effects and interactions. A p value of <0.05 was used to determine significance.

5.2.4. *Results:*

Results from the MAO-A and -B assays clearly show that animals treated with VIG were no different than animals treated with VEH in terms of their inhibition of these enzymes (Figure 27). Animals treated with PLZ on the other

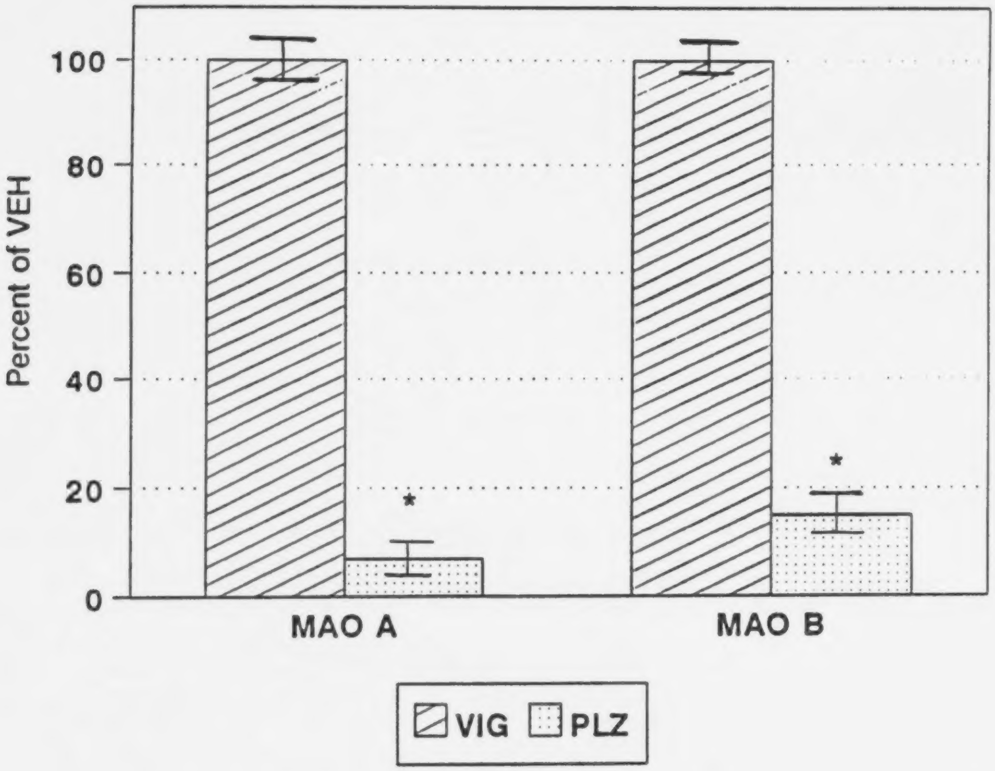

Figure 27. MAO-A and -B activity from rats treated with VIG (1000 mg/kg) or PLZ (10 mg/kg) (n=6). Histograms represent percent of VEH ± SEM. * Denotes significantly different from VEH.

hand showed, as expected, a marked inhibition of both MAO-A and -B (93 and 85% respectively).

Figure 28 displays the data obtained from assaying brains from all groups for GABA content. Both VIG and PLZ treatments resulted in an increase in GABA levels of almost 3-fold. There was not a significant difference between the levels of GABA in the VIG-treated animals as compared to the PLZ-treated animals ($p>0.05$).

The data from the GABA-T enzymatic analysis are shown in Figure 29. Animals treated with VIG showed a mean inhibition of GABA-T of 42%. The mean inhibition of GABA-T in animals treated with PLZ was 30%.

Figure 30 is a graphic representation of the data obtained from assaying the brains from all groups for ALA levels. PLZ resulted in a significant increase in ALA compared to the VEH and VIG-treated animals ($p<0.05$).

The results of the ALA-T enzymatic analysis is shown in Figure 31. Only PLZ treatment resulted in significantly reduced ALA-T activity as compared to the VEH control animals. The mean inhibition of ALA-T in PLZ-treated animals was 28%.

5.2.5. Part 2. Drug Combination

5.2.6. Drug Administration and analysis:

Male Sprague Dawley rats weighing 250-300 g with access to food and water *ad libitum* were randomly assigned ($n=5$-6) to one of the following

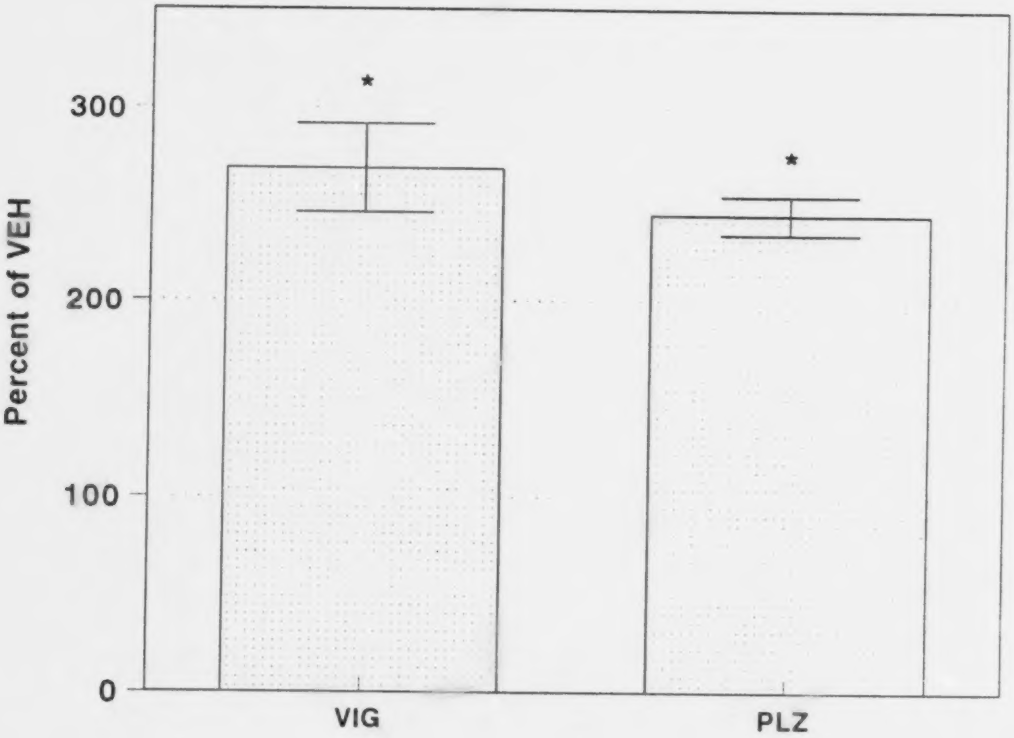

Figure 28. Brain GABA levels from rats treated with VIG (1000 mg/kg) or PLZ (10 mg/kg) (n=6). Histograms represent percent of VEH ± SEM. * Denotes significantly different from VEH (mean control levels were 295.18± 28.4 μg/g tissue).

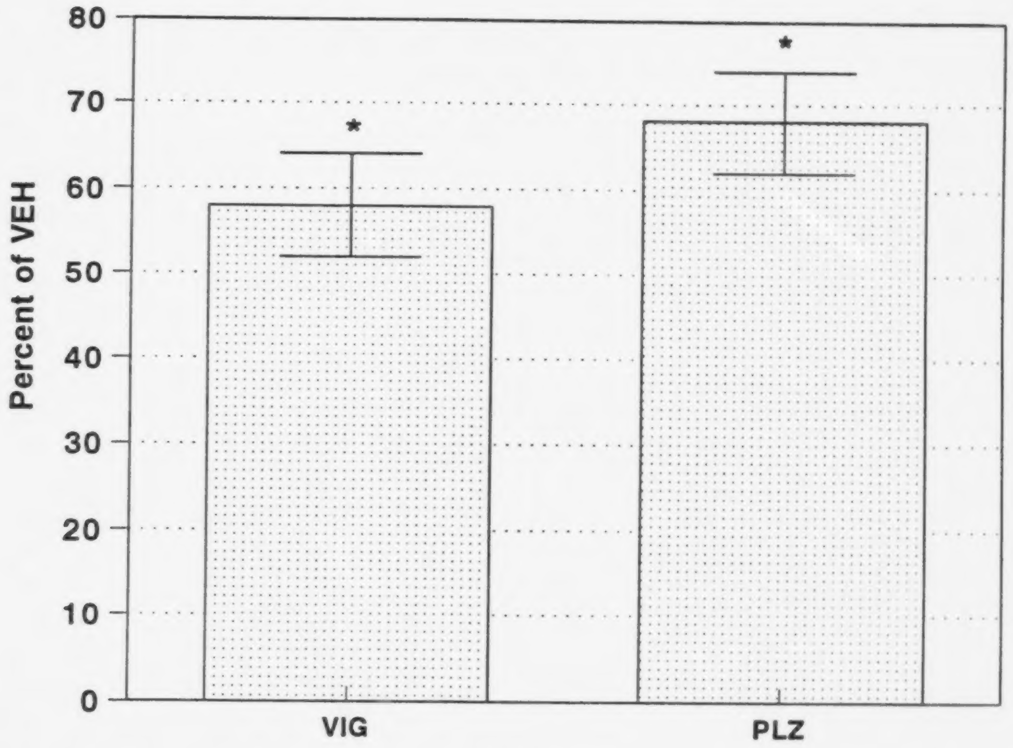

Figure 29. Brain GABA-T inhibition from rats treated with VIG (1000 mg/kg) or PLZ (10 mg/kg) (n=6). Histograms represent percent of VEH ± SEM. * Denotes significantly different from VEH.

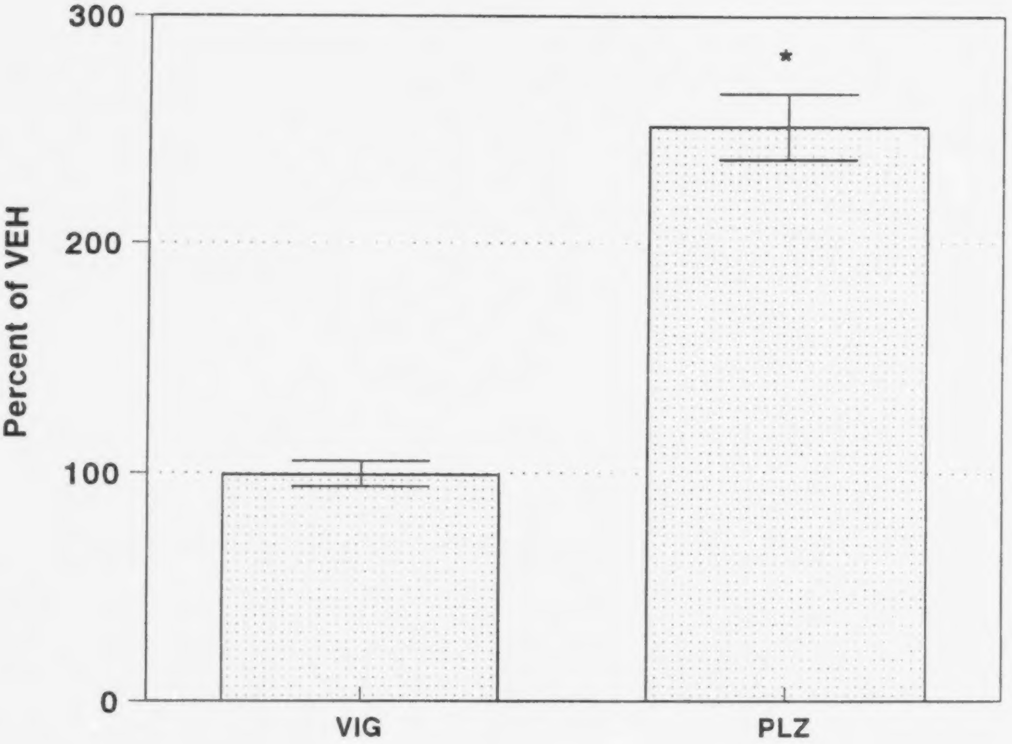

Figure 30. Brain ALA levels from rats treated with VIG (1000 mg/kg) or PLZ (10 mg/kg) (n=6). Histograms represent percent of VEH ± SEM. * Denotes significantly different from VEH (mean control levels were 55.38± 3.8 μg/g tissue).

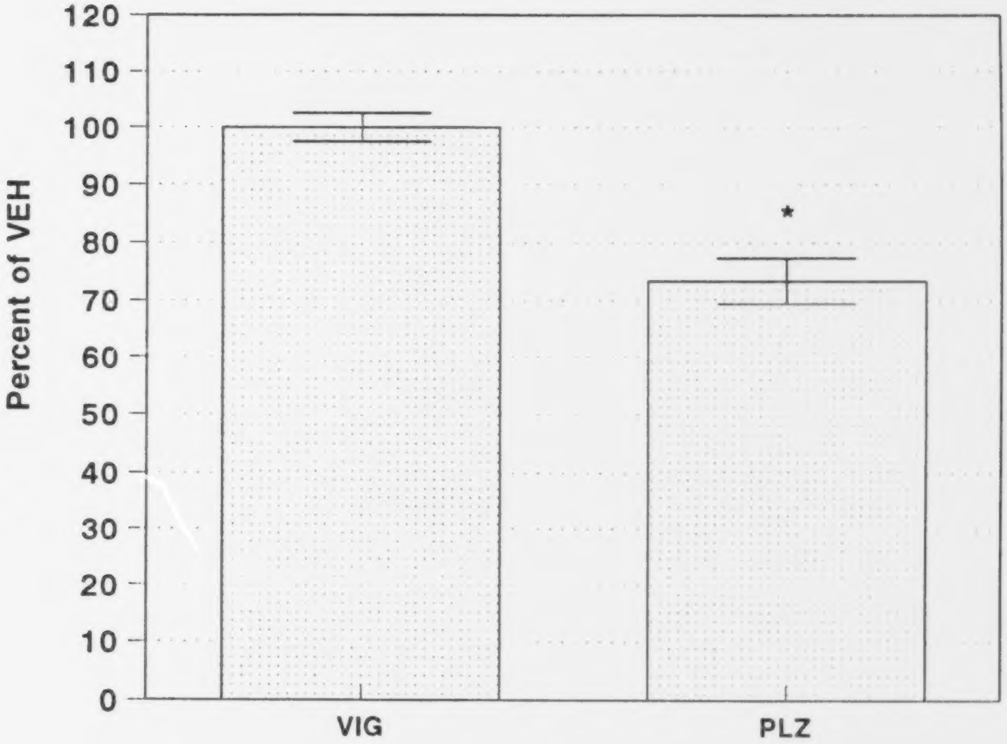

Figure 31. Brain ALA-T inhibition from rats treated with VIG (1000 mg/kg) or PLZ (10 mg/kg) (n=6). Histograms represent percent of VEH ± SEM. * Denotes significantly different from VEH.

groups; TCP/VIG, TCP/PLZ, VEH/VIG or VEH/VEH. Previously, control groups were run for the control combinations of TCP/VEH and VEH/PLZ and these data are included here for comparison. Doses for VIG and PLZ (1000 mg/kg and 10 mg/kg respectively) were the same as in Part 1 while the dose of TCP was 1 mg/kg.

Rats were injected IP with TCP or VEH depending upon their group assignment 1 h prior to injection of VIG, PLZ or VEH, again dependent upon group assignment. As in Part 1, all injections were administered in a counterbalanced fashion. Four hours after the second injection, the animals were sacrificed by decapitation and the brains treated in the same manner described in Part 1.

5.2.7. Results:

GABA levels are represented in Figure 32. The VEH/VEH, TCP/VEH and TCP/PLZ treated groups were not significantly different from each other, with values ranging from 230 to 260 μg/g tissue. The animals treated with VEH/VIG, VEH/PLZ and TCP/VIG were not significantly different from each other (values ranging from 612 to 660 μg), but had GABA levels significantly higher than the VEH/VEH and TCP/VEH control groups and the TCP/PLZ treated animals (interaction $F(1,29)=7.89$ $p<0.05$). In other words, TCP completely blocked the GABA-elevating action of PLZ, but had no effect on the GABA-elevating action of VIG.

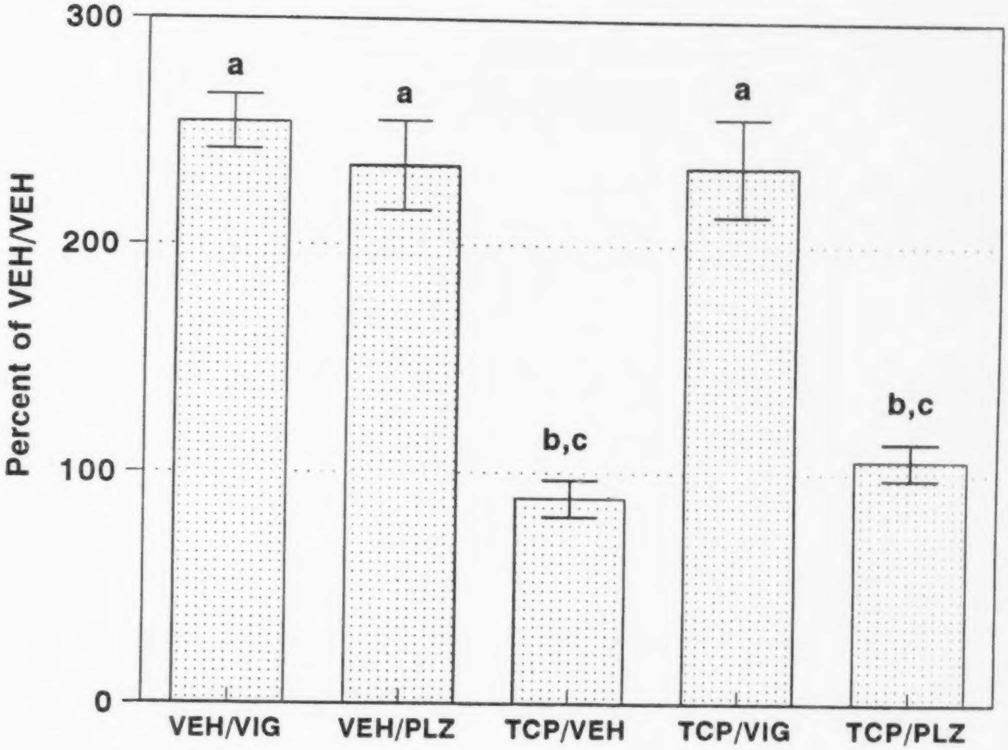

Figure 32. Brain GABA levels in rats treated with drug combinations (VIG 1000 mg/kg; PLZ 10 mg/kg; TCP 1 mg/kg, n=6). Histograms represent percent of VEH/VEH ± SEM. a, significantly different from VEH/VEH; b, significantly different from VEH/VIG; c, significantly different from VEH/PLZ (VEH/VEH control values were 230.77± 20.4 μg/g tissue).

Results from the GABA-T analysis are shown in Figure 33. These data coincide nicely with the results from the GABA levels in that only the groups treated with either VEH/VIG, VEH/PLZ or TCP/VIG had significantly inhibited GABA-T activity. There were no significant differences between any of the other groups (TCP/VEH and TCP/PLZ) compared to the VEH/VEH control group (interaction $F(1,24) = 6.05$ $p<0.05$). Therefore, pretreatment with TCP reversed the action of PLZ on GABA-T but had no effect on the GABA-T inhibiting properties of VIG.

The results of the analysis of brain ALA levels are represented in Figure 34. Only the VEH/PLZ group had significantly elevated ALA levels compared to the VEH/VEH control group (interaction $F(1,29) = 7.95$ $p<0.05$).

The data obtained on ALA-T activity for the drug combination treatments are displayed in Figure 35. These results showed that only the VEH/PLZ group resulted in a significant decrease in ALA-T activity as compared to the VEH/VEH control group (interaction $F(1,24)=6.47$ $p<0.05$).

5.3. DISCUSSION

5.3.1. Part 1. Single drug treatment

The results from this investigation comparing the *ex vivo* effects of VIG and PLZ revealed, not unexpectedly, that VIG has no effect on MAO. VIG has been reported to have no effect on other oxidative enzymes such as succinic

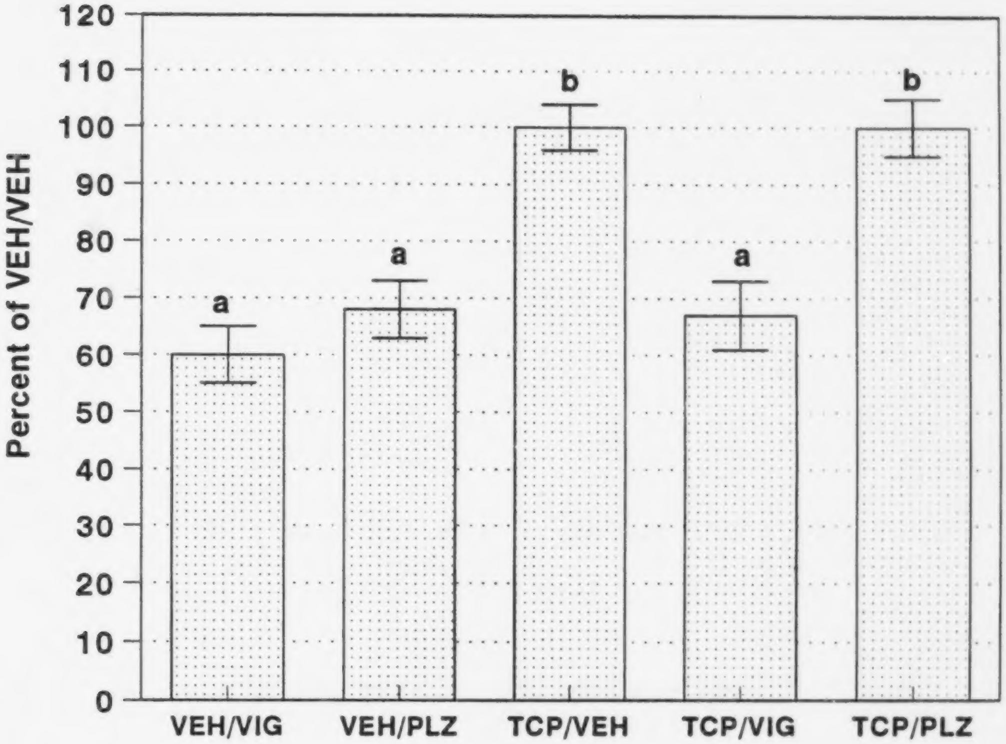

Figure 33. Brain GABA-T inhibition from rats treated with drug combinations (VIG 1000 mg/kg; PLZ 10 mg/kg; TCP 1 mg/kg, n=6). Histograms represent percent of VEH/VEH activity (± SEM). a, significantly different from VEH/VEH; b, significantly different from VEH/PLZ.

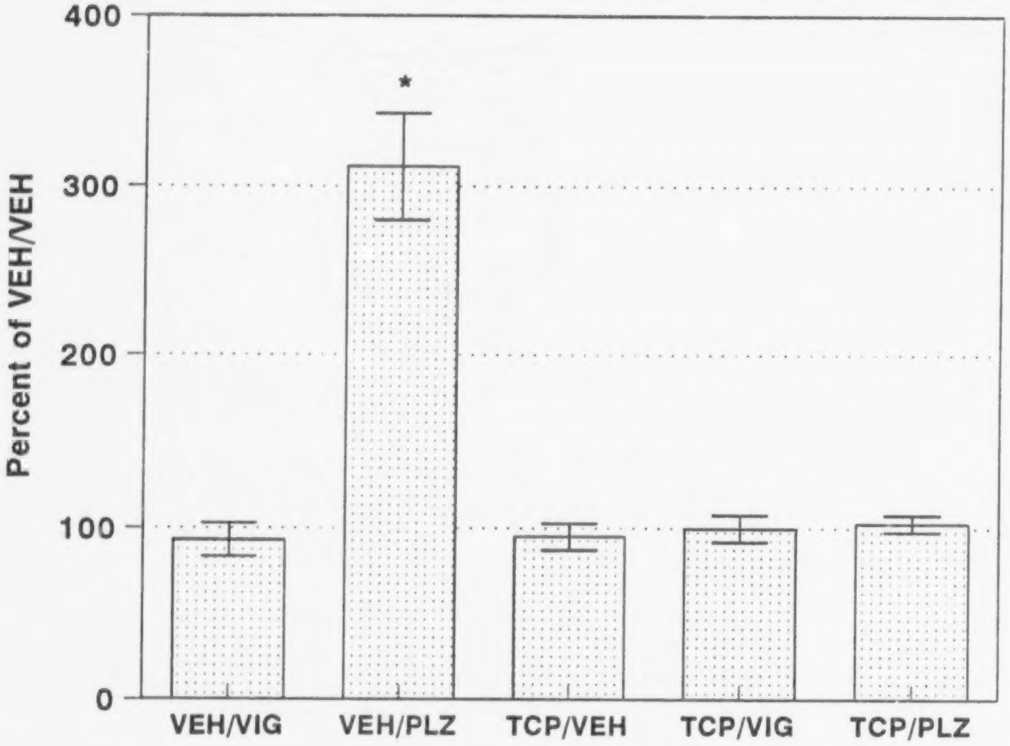

Figure 34. Brain ALA levels in rats treated with drug combinations (VIG 1000 mg/kg; PLZ 10 mg/kg; TCP 1 mg/kg, n=6). Histograms represent percent of VEH/VEH ± SEM. * Denotes significantly different from VEH/VEH. (VEH/VEH control values were 55.3± 3.4 μg/g tissue).

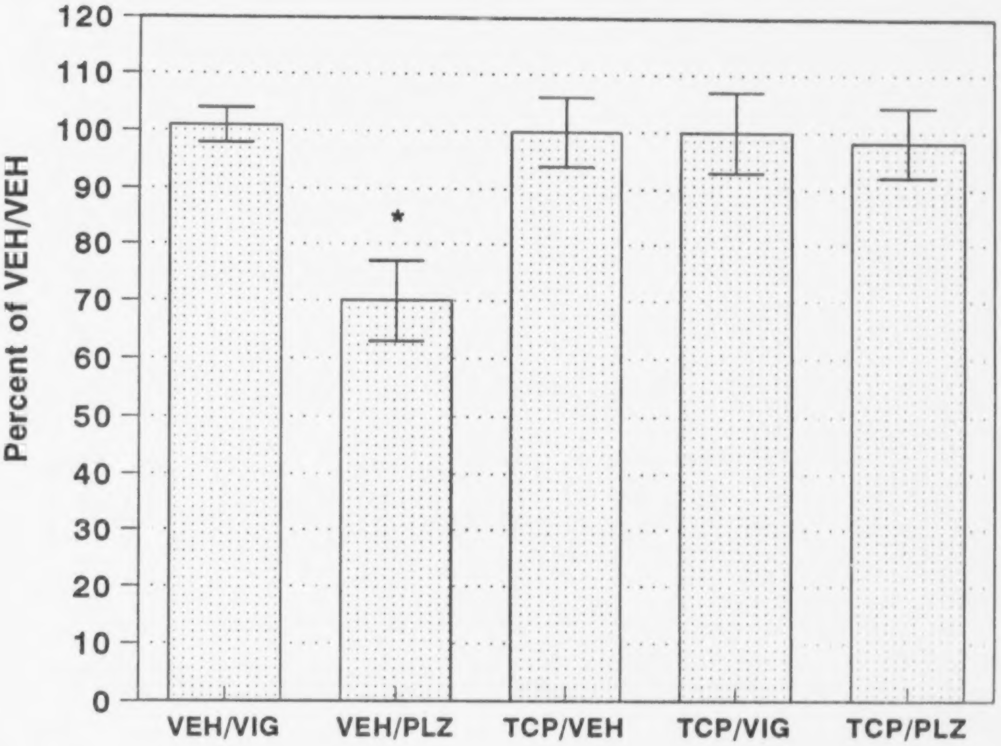

Figure 35. Brain ALA-T inhibition from rats treated with drug combinations (VIG 1000 mg/kg; PLZ 10 mg/kg; TCP 1 mg/kg, n=6). Histograms represent percent of VEH/VEH activity (± SEM). * Denotes significantly different from VEH/VEH.

semidaldehyde dehydrogenase (Jung, 1977). Additionally, it was reported that VIG was fairly specific to GABA-T in that it did not inhibit other transaminases such as aspartate transaminase and liver ornithine transaminase (Jung, 1977).

The present investigation also found that VIG (at the dose tested), unlike PLZ, had no effect on the activity of ALA-T. The effects of PLZ on the inhibition of MAO-A and MAO-B found in the present study were consistent with other results presented earlier in this thesis.

At the doses tested, both VIG and PLZ resulted in similar elevations of GABA levels in rat brain and a similar degree of inhibition of GABA-T. It is interesting to note, that in order to produce as large an increase in levels of the catecholamines and/or 5-HT as GABA levels observed here, the degree of MAO inhibition by PLZ needs to be much greater than the inhibition of GABA-T. A 2-3 fold increase of GABA levels is observed with PLZ at a dose which produces 30% inhibition of GABA-T, while it has been proposed (Ravaris *et al.*, 1976) that approximately 85% inhibition of MAO is required before significant elevation of brain levels of catecholamines or 5-HT is observed.

5.3.2. Part 2. Drug Combinations

The results obtained from the GABA level analysis showed that prior treatment with TCP blocked the GABA-elevating effects of PLZ, but not of VIG even though PLZ and VIG are apparently both inhibitors of GABA-T. Popov and Matthies (1969) found a similar pattern when comparing the effects of pretreatment of TCP on the GABA-elevating actions of PLZ and the GABA-T inhibitor aminooxyacetic acid, that is, TCP reversed the action of PLZ, but had no effect on aminooxyacetic acid. These observations further support the supposition that it is a metabolite produced by the action of MAO on PLZ that is responsible for the GABA-elevating effect, at least in the acute situation. Prior treatment with TCP also blocked PLZ's inhibitory effects on the enzyme GABA-T, but had no significant effect on the inhibition of GABA-T by VIG.

In the combination treatments, only the VEH/PLZ group showed significantly elevated ALA levels. Similarly, with regard to ALA-T inhibition, only the VEH/PLZ showed significantly reduced ALA-T enzymatic activity. Currently, there is little known regarding the role of ALA in the central nervous system or its possible involvement in the etiology of depression or panic disorder. It has been reported that administration of the convulsant pentamethylenetetrazole increases brain ALA levels (Clarke *et al.*, 1989). As discussed in Chapter 4 of this thesis, ALA is metabolically related to lactate, which has been shown, in some patients, to produce panic attacks (Shear, 1986). The antipanic effects of PLZ then may be due, in part, to a reduction in the formation of lactate *via* the inhibition of ALA-T.

Taken together, the results from both experiments suggest that the GABA-elevating effects of PLZ are similar to those of VIG and seem to be due, at least in part, to the inhibition of GABA-T. While PLZ is also an inhibitor of ALA-T and increases brain ALA levels, VIG has no such effect. Elevation of brain levels of both GABA and ALA by PLZ can be blocked by the prior treatment with the MAO inhibitor TCP. This latter finding supports the notion that it is likely not PLZ that is responsible for the inhibition of GABA-T and ALA-T elevation of brain GABA and ALA levels, but a metabolite of PLZ produced by the actions of MAO on PLZ.

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6.1 GENERAL DISCUSSION

Results presented in Chapter 2 of this thesis are in conflict with a previous report that BZD binding sites are down-regulated following chronic antidepressant treatments (Suryani-Cadotte *et al.*, 1985). They concur, however, with other investigations that reported no change in BZD binding sites (Lloyd and Pilc, 1984; Kimber *et al.*, 1987). Lending weight to the argument against BZD down-regulation being an important component of antidepressant treatment in the present study is the finding of a down-regulation of 5-HT$_2$ binding in the *same* rat brains. Previous reports have shown that down-regulation of 5-HT$_2$ receptors is a property shared by several antidepressants (Baker and Greenshaw, 1989; Eison *et al.*, 1991). The present investigation is the first to measure both BZD and 5-HT$_2$ binding as well as the levels of the drugs, subsequent to chronic antidepressant treatment, in the same rat brain. The resultant data strongly suggest that changes in the number or affinity of the BZD binding sites are not involved in antidepressant action.

Attention was then directed towards the GABAergic effects of the MAO inhibitor PLZ. Investigations described in Chapter 3 showed that administration of PLZ, a nonselective MAO inhibitor, resulted in a dramatic increase in brain GABA levels. This increase was effectively blocked by a preinjection of another nonspecific MAO inhibitor, TCP. Preinjection of the selective MAO-B inhibitor DEP was more effective than the selective MAO-A inhibitor clorgyline at blocking the GABA-elevating effects of PLZ. These data suggest that MAO-B may play a more important role in the GABA-elevating effects of PLZ than MAO-A.

It was also of interest to ascertain whether the elevation of GABA was unique to PLZ, or a property common to hydrazine-containing MAO inhibitors. Previously, N^2-acetyl-PLZ was found to have no effect on either GABA or ALA levels (McKenna *et al.,* 1991). The present investigations considered the neurochemical effects of varying doses and times of IPR and NIAL, two nonspecific MAO inhibitors containing substituted hydrazine functions. Although both drugs produced a dose-dependent inhibition of MAO (and subsequently increased biogenic amine levels), neither had any effect on GABA, ALA or their catabolic enzymes. These investigations clearly show that a free hydrazine group is critical for elevation of brain GABA and ALA but not for MAO inhibition.

Currently, GABA is considered to play a role not only in depression, but also in panic disorder (Breslow, 1989). Specifically, increasing functional GABA levels seems to have positive effects in patients with panic disorder. Comparing PLZ to VIG, a known GABA-elevating drug (*via* blockade of GABA-T) on a number of parameters was of interest. The results from this series of studies showed that TCP blocked the GABA-elevating effect of PLZ but had no effect on GABA-elevation by VIG. TCP also reversed the action of PLZ on GABA-T, but had no effect on inhibition of GABA-T by VIG. These data support the supposition that it is not PLZ *per se* that is responsible for the inhibition of GABA-T and elevation of GABA levels, but that it is more likely that these actions are due to the formation of a metabolite produced by the actions of MAO on PLZ. In the same study, only PLZ was shown to inhibit ALA-T and result in significantly increased brain ALA levels. Although VIG has proven effective in seizure control, its effectiveness in panic disorder has yet to be delineated (Kalviainen *et al.,* 1993). ALA is related metabolically to lactate which is known to provoke panic attacks in some individuals (Shear, 1986). It is possible that the alleviation of panic symptoms some patients experience following treatment with PLZ could be due, at least in part, to the inhibition of ALA-T and a related reduction in the formation of lactate.

In summary, a number of issues pertaining to the GABAergic mechanisms involved in the actions of the antidepressant/antipanic drug PLZ have been addressed in this thesis. Also, a comprehensive research project was conducted in an attempt to clarify the controversy regarding BZD binding site changes following chronic antidepressant treatments. As discussed in this thesis, further explanations of this controversy will probably require the use of molecular biological techniques.

6.2 POSSIBLE FUTURE RESEARCH

The results described in this thesis have revealed some potentially important areas of research with regard to understanding more fully the mechanisms of action of PLZ. Studies on the effects of PLZ on the enzymes glutamine synthetase and on the uptake of GABA into nerve terminals and glia may help explain the marked increases in brain levels of GABA produced by PLZ. It would also be of interest to investigate the effects of PLZ on brain lactate, since this acid may play a role in the antipanic effects of PLZ. Obviously, further research should be conducted in an effort to establish the identity of the metabolite(s) of PLZ which is/are contributing to the elevating effects of this drug on GABA and ALA in brain. It is certainly possible that PLZ's actions are the results of a combination of factors, and studies designed to manipulate each of these factors independently would be of great value.

6.3 REFERENCES

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